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# High Throughput Design of Functionalized Nanoparticles for Targeted Vaccine Delivery

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**High throughput design of functionalized nanoparticles for  
targeted vaccine delivery**

by

**Ana Vianey Chavez Santoscoy**

A thesis submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of  
MASTER OF SCIENCE

Major: Chemical Engineering

Program of Study Committee:  
Balaji Narasimhan, Major Professor  
Surya Mallapragada  
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Iowa State Univesity

Ames, Iowa

2011

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*To my family, who taught me that even the greatest goal can be accomplished if  
it is done one step at a time*

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## LIST OF SYMBOLS AND ACRONYMS

$^1\text{H}$ NMR	Proton nuclear magnetic resonance
AFM	Atomic force microscopy
AM□	Alveolar macrophage
APC	Antigen Presenting Cells
BAL	Bronchoalveolar lavage
BALT	Bronchus-associated lymphoid tissue
BMM□	Bone-marrow derived macrophages
CD40, CD86, CD4, CD8	Clusters of differentiation
CIRE	Murine homologous of DC-SIGN. See DC-SIGN
CLR	C-type lectin receptor
CPH	1,6-bis(p-carboxyphenoxy)hexane
CPTEG	1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing non-integrin
DLS	Dynamic light scattering
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride)
FA	Fumaric acid
FDA	Food and Drug Administration
GPC	Gel permeation chromatography
HA	Hemagglutinin
IFN- $\gamma$	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL -1 $\beta$ , IL-6, IL-12, IL-12p40	Interleukins
KO	Knockout
LPS	Lipopolysaccharide
MALT	Mucosal-associated lymphoid tissue
MGL	Macrophage Galactose Lectin
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
MMR	Macrophage Mannose Receptor
$M_w$	Molecular weight
M□	Macrophage
NA	Neuraminidase
NALT	Nasal-associated lymphoid tissue
NF- $\kappa$ B	Nuclear factor kappa B
NHS	N-hydroxysuccinimide
PAMP	Pathogen-associated molecular patterns
PCS	Photon correlation spectroscopy
PEG	Polyethylene glycol
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)

PLGA	Poly(lactic-co-glycolic acid)
PRRs	Pattern recognition receptors
QD	Quantum dot
RLR	RIG-I-Like Receptor
RNS	Reactive Nitrogen Species
SA	Sebacic acid
SEM	Scanning Electron Microscopy
TEM	Transmission electron microscopy
T <sub>g</sub>	Glass transition temperature
Th	Helper T cells
TLR	Toll-like receptor
TNF- $\alpha$	Tumor necrosis factor alpha
TT	Tetanus toxoid
WHO	World Health Organization
WT	Wild type

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## **CHAPTER 1: INTRODUCTION**

### **1.1 Introduction**

Vaccination is one of the most important accomplishments of medicine, resulting in a cost-effective strategy for controlling infectious diseases [1]. As stated by the vaccinologist, Stanley Plotkin, the impact of vaccination has been enormous in terms of mortality reduction, even greater than antibiotics [2]. Vaccination has made possible the eradication of smallpox, the elimination in certain regions of the world of infectious diseases such as measles and poliomyelitis, and the reduction of morbidity and mortality caused by diseases as tetanus and diphtheria [1]. However, acute respiratory infections, such as pneumonia and influenza, are only partially controlled by vaccines [3]. In addition, the threat of bioterrorism attacks using bioweapons or pathogens [4, 5] has added a sense of urgency to the effort to improve safety and efficacy of vaccines [6]. The emergence of new antigens, including recombinant proteins which are poorly immunogenic, is generating the need to design novel and more potent immune adjuvants for the development of efficacious vaccine formulations.

Influenza has recently gained significant attention because of the possibility of rapidly emerging strains that might cause a pandemic outbreak. The 1918-19 pandemic, also referred to as “Spanish flu”, caused at least 20 million deaths [7]. In addition, recent concerns due to the advent of pathogenic H5N1 strains, which have been responsible for higher mortality rates than anticipated, have led to the thought that a pandemic strain of influenza is likely to emerge in the near future [8]. As a second example, pneumonia is the leading cause of death in infants under five years old. This is a disease that is

aggravated by poverty, malnutrition, air pollution, and the presence of other infectious diseases. It is also a serious problem for adults over 65 years old [3]. Other agents that represent a potential problem because of their likelihood to be used as bioterrorism agents include anthrax, plague, Q fever, and tularemia [9].

Advances in pharmaceutical and biotechnological research have resulted in the discovery and creation of new biomolecules, including recombinant proteins and nucleic-acid-based molecules, as promising therapies and treatments. To keep pace, there needs to be a dramatic increase in efforts to develop new delivery technologies. The main objective of these efforts is to overcome challenges such as: protein stability and preservation of bioactivity, protection against enzymatic degradation and pH instabilities, and controlled release of the therapeutic agent at physiological conditions [10, 11].

In the vaccination area, recent efforts have focused on the production of recombinant proteins as specific antigens. These technologies have emerged from the need to look for alternatives to live or killed whole organism vaccines, which generally had safety issues (e.g., local and systemic reactions such as inflammation, granulomas, ulcers, necrosis, etc.) [12]. However, this new generation of technologies poses challenges not only in the development of these molecules, but also in terms of maintaining their immunogenic potency and mechanisms to administer them. The need for effective and safe delivery devices with adjuvant properties is clear and it has driven the rational design of vaccine formulations utilizing biodegradable materials, including polyesters and polyanhydrides, among other technologies, as adjuvants and delivery vehicles [13-15].

In addition to adjuvanticity, biocompatibility, and controlled release, targeting the vaccine to immune cells has emerged as a viable and powerful strategy. In this context, potent adjuvants are being developed utilizing two main targeting strategies: targeted delivery (i.e., controlling the site and time of release, as well as the fate of the delivery system according to physical conditions – such as size and chemistry) and targeting of receptors (i.e., controlling immunological stimulation by targeting specific receptors).

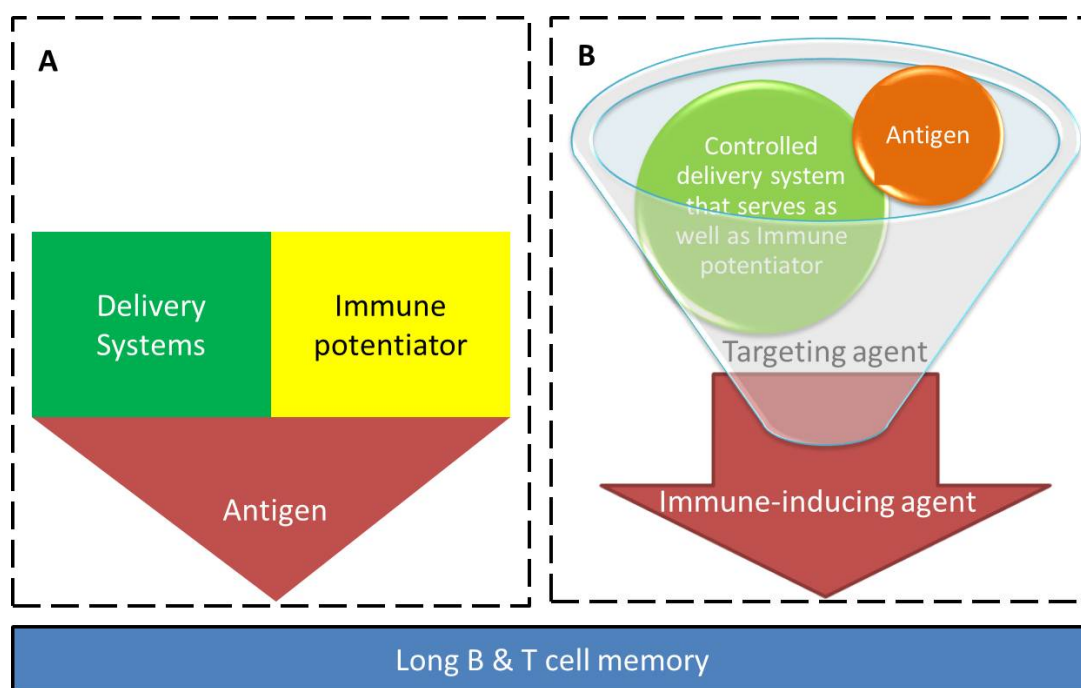
It has been proposed that the components of effective vaccines are: a delivery system, an immune potentiator or adjuvant, and an antigen [16]. In this regard, polyanhydride particles have emerged as an important platform, capable of performing the dual functions of serving as an adjuvant and as a delivery system. Recently, Ulery and co-workers reported on a vaccine formulation that included the integration of a controlled delivery system that also served as an immune potentiator (an amphiphilic polyanhydride chemistry), an immune-inducing agent (soluble antigen), and an antigen (encapsulated antigen) [17]. The main goal of this thesis is to confer targeting properties to this vaccine formulation that directs the previously mentioned components towards a potent and long lasting immune response (**Figure 1.1**). In other words, the selection of targeting agents (depending upon the specific pathogen) would be key to enhance and direct the immune response in a specific manner to achieve protection.

There is a critical need for the development of efficacious vaccines against respiratory pathogens capable of inducing robust and protective immune responses at mucosal surfaces. Intranasal application of vaccines can trigger an appropriate immune response at systemic and mucosal sites. Intranasal vaccination promotes a more effective, earlier, and stronger immune response in comparison to other routes of

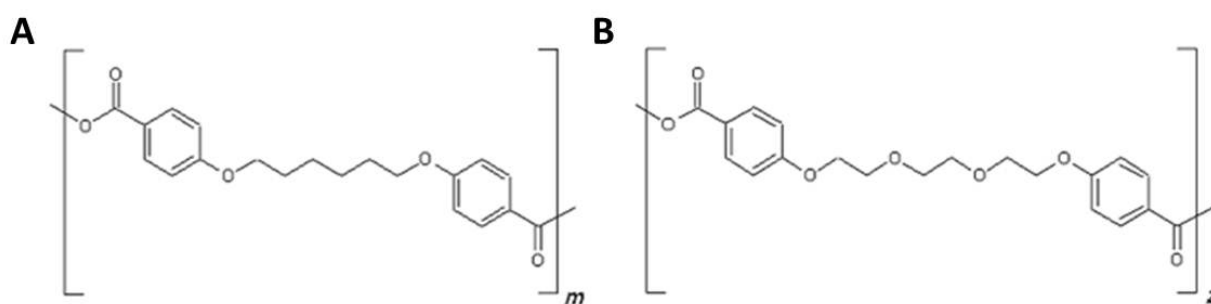
administration [18]. Since this dissertation focuses on intranasal vaccines against respiratory pathogens, alveolar macrophages were the main subject of study; these cells are the key immune cells in the lung immunity (see Chapter 2, Section 2.3).

Polyanhydride nanoparticles are promising vaccine platforms that have the potential to be used as controlled drug delivery devices as well as potent and effective immune adjuvants for the design of single dose vaccines [15, 19, 20]. It has been shown that changing the chemistry of these nanoparticles can change their uptake by immune cells and the resultant immune response can be tailored [21, 22]. In addition their pathogen mimicking properties have been investigated [17]. Based on these studies, new strategies can be exploited in the rational design of these adjuvants, mimicking in a more direct manner how immune cells recognize pathogens. Targeting pattern-recognition receptors (PRRs) (i.e., C-type lectin receptors or CLR) provides a valuable tool to improve internalization, antigen presentation, and depending on the target, enhancement of the immune response.

The overall goal of this project is to design a targeted nanovaccine platform by functionalizing the surface of amphiphilic polyanhydride nanoparticles to target specific C-type lectin receptors (CLR). This study focuses on amphiphilic polyanhydrides based on 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) (**Figure 1.2**). The saccharides used to modify the surface of CPTEG:CPH nanoparticles are di-mannose and galactose.



**Figure 1.1 Components of effective vaccines.** (A) represents the components of an effective vaccine, including a delivery system, an immune potentiator or adjuvant and antigen (part A of the figure was adapted but modified from O'Hagan 2007 [16]). (B) represents the proposed platform for the rational design of vaccines that integrates a controlled delivery system and an immune potentiator, an immune-inducing agent, an antigen and a targeting agent to enhance the efficacy of vaccines.



**Figure 1.2 Polyanhydride chemical structures.** Chemical structures of (A) 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) and (B) 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG).



## 1.2 Dissertation Organization

**Chapter 2** presents a literature review in the areas of on hydrolytically degradable polymers used as vaccine adjuvants and delivery systems, immunological aspects of vaccine design, and current targeting strategies. **Chapter 3** presents the overall research goals of this project. The development of high throughput methods to fabricate novel polyanhydride nanoparticles with a targeting ligand (i.e., carbohydrates to target C-type lectin receptors) is presented in **Chapter 4**. The interaction between nanoparticles and alveolar macrophages (AM $\square$ ) and the role of surface functionalization on the internalization of nanoparticles and further activation of AM $\square$  is presented in **Chapter 5**. **Chapter 6** concludes the thesis with some directions for future research.

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## CHAPTER 2: BACKGROUND AND LITERATURE REVIEW

### 2.1 Introduction

This dissertation focuses on the design of nanoparticles modified with carbohydrates, which can potentially be used as targeted adjuvants for intranasal vaccines. This chapter is organized into the following sections: biodegradable polymers used as vaccine adjuvants and delivery systems (**Section 2.2**), immunological aspects of vaccine design (**Section 2.3**), and targeted vaccine design (**Section 2.4**).

Polymeric particles have been used as delivery devices for drugs and bioactive molecules. The efficacy of these devices depends on the interaction of the polymer with the payload as well with the biological environment. Investigations have focused on the effect of chemistry, size, surface charge, and erosion mechanism on the delivery of many agents. In particular, for the design of intranasal vaccines, only a few biodegradable polymers have been investigated. The rational design of vaccines requires an in-depth understanding of the immune system, including the connection between innate and adaptive immunities.

Pathogen mimicking strategies for vaccine design include the rational design of the vaccine formulation so it can elicit a similar immune response to the one generated by the pathogen but without the non-desirable effects of the infection. Selecting the route of immunization is fundamental, so that the immune response can be generated at the site of infection, or in other words, where the pathogen is more likely to be encountered. For respiratory pathogens, intranasal vaccines are promising for the

generation of local and systemic immunity. Engineering intranasal vaccines requires a comprehension of the lung immunity including its regulatory mechanisms.

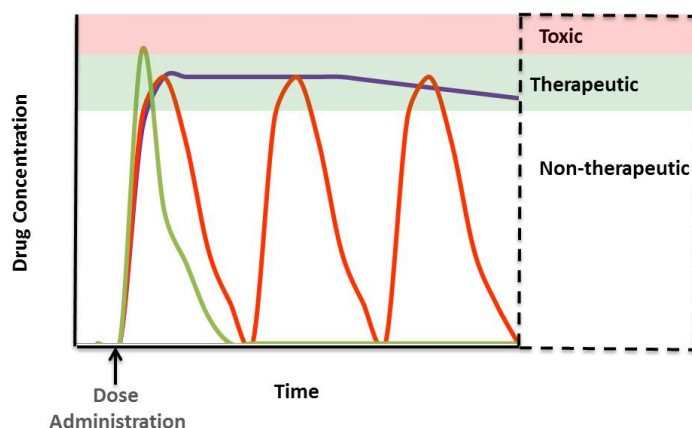
## **2.2 Biodegradable polymers as delivery systems**

### **2.2.1 Introduction**

Biodegradable polymer-based microparticles (diameter  $> 1\mu\text{m}$ ) and nanoparticles (diameter  $< 1\mu\text{m}$ ) have been used as delivery devices in order to improve the therapeutic value of various drugs and bioactive molecules [1, 2]. Biodegradable polymers include hydrolytically degradable polymers as well as enzymatic degradable polymers, which may be synthetic or natural [3]. The goal is to overcome several challenges including preservation of stability, improved bioavailability, preservation of bioactivity and biocompatibility, protection against enzymatic degradation and pH instabilities, targeted delivery to specific cells and or tissues enabling transport across biological barriers, and enhancement of the retention time and controlled release [1, 4-13]. The performance of particulate devices as drug delivery carriers depends on particle size, surface and matrix chemistry, and their interactions with the payload and biological systems [1, 2, 14].

A successful delivery vehicle has a high loading capacity to reduce the amount of the carrier required for administration [1, 15]. In addition, controlled release of a drug or bioactive molecule is desirable to reduce frequency of doses and maintain appropriate concentrations at local and systemic levels [16]. Controlled release may be pulsatile or continuous and **Figure 2.1** shows these release profiles in comparison with

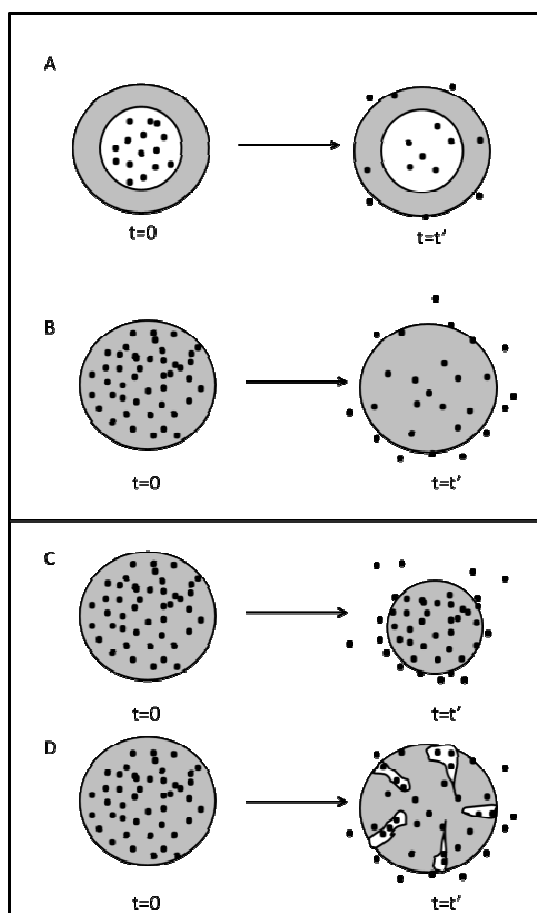
conventional administration. The use of biodegradable polymers is more efficient for the continuous release of a drug/bioactive molecule over time.



**Figure 2.1 Release profile schemes.** Conventional release (—) of a drug or bioactive molecule requires multiple doses in order to maintain the drug concentration in the therapeutic range (■). In this case, the drug concentration rapidly falls into the non-therapeutic range (□). If the initial serum concentration of the drug is too high, initial concentration of the drug is avoided in order to prevent the systemic concentration may become toxic (■). Controlled release devices are designed so that the release can be continuous (—) or pulsatile (—), in order to maintain the drug concentration within the therapeutic range. Modified and adapted from Brannon-Peppas [16].

There are three mechanisms of incorporating a drug/bioactive molecule into polymeric particles: loading at the time of nanoparticle fabrication [17-19], by linkage to the surface [20, 21], or by absorption on the surface after fabrication [22]. The use of each method depends on the chemistry of the therapeutic agent and the polymer, and the incorporation conditions [1, 23]. Depending on how the drug/bioactive molecule is contained, the entity is referred to differently. If the drug/bioactive molecule is located in a compartment surrounded by a polymer matrix, it is called a capsule. In the case that the molecule is dispersed in the polymer matrix, it is called a particle [1, 24]. Depending on the incorporation mechanism and the chemistry of the matrix polymer, the release

profiles may be different. When the drug is incorporated by loading in the fabrication method, the release profile depends on the erosion mechanism of the polymer and the size of the particle. A degradable polymer might undergo surface, bulk, or combined erosion profiles (**Figure 2.2**) [2, 25].



**Figure 2.2 Release mechanisms.** Diffusion is the main release mechanism either from a compartment that contain the therapeutic agent surrounded by a matrix, called capsule (A), or from a dispersed state in the polymer, called particle (B), to the exterior. Biodegradable can degrade by two different mechanisms: Surface erosion (C) where the surface to degrade layer-by-layer, and bulk erosion (D) where water penetrates the device quickly allowing for the dissolution of the material. Initial time is represented by  $t=0$  and a later time is represented by  $t'$ . Modified and adapted from Langer [2].

The release profile differs depending on the erosion mechanism. When solvent (*i.e.*, water at physiological conditions) penetrates into the matrix of the particle (bulk erosion), an increased “burst release” and usually a more rapid release kinetics is likely to occur [26]. Size is another important variable that affects the release of the drug or bioactive molecule from particles. Nanoparticles offer more surface area than microparticles, which provides more surface area for contact with water, and hence nanoparticles are more likely to aggregate [26].

The rational design of a drug delivery device includes the selection of the chemistry of polymer, not only to determine release profiles, but also for safety issues, since there are concerns about the safety of certain degradable polymers because high concentrations of degradation products might be toxic or cause instabilities to the therapeutic agent incorporated into the particle [24]. Biocompatibility is a term that has evolved from the understanding of an biological inert material to a material that interacts with the biological environment in an appropriate or beneficial manner depending on the specific application [27]. In particular, for the design of vaccines, the biocompatibility of the polymeric material and the degradation products is a very important characteristic in order to design not just effective, but safe formulations. For the rational design of vaccines, an in-depth understanding of biocompatibility is needed as the interaction of the material with its biological surroundings may result in immunomodulatory or adjuvant characteristics, but should not cause toxic effects on the host. The selection of polymers for rational vaccine design should take into consideration these characteristics.



### **2.2.2 Microparticles and nanoparticles**

The selection of the particle size is important depending on the specific application. Size has an important effect on the fate of the particle. Usually nanoparticles distribute more rapidly through the body (independent of the route of administration), but are more rapidly cleared as well; in contrast, microparticles tend to stay where they are placed [28-30]. For example, studies have shown that slow-degrading microparticles were found at the site of infection after more than 8 weeks; in contrast, nanoparticles were cleared completely in the same time [31]. However, nanoparticle clearance can be prevented or delayed by reducing nonspecific distribution and targeting specific tissues or cell receptors by incorporating targeting ligands (peptide, aptamer, antibody, carbohydrates and other small molecules) [32]. Nanoparticles are more effective traveling through capillary regions and the respiratory track. This characteristic is useful to target specific organs such as lymph nodes or liver [33, 34], or for specific applications like intranasal administration [35, 36] or brain delivery [37, 38]. The diffusion of the drug is also affected by the particle size. Presumably, a greater proportion of drug can leave the surface of drug-loaded nanoparticles (which are smaller entities than microparticles) by diffusion [26].

#### **2.2.2.1 Fabrication and characterization methods**

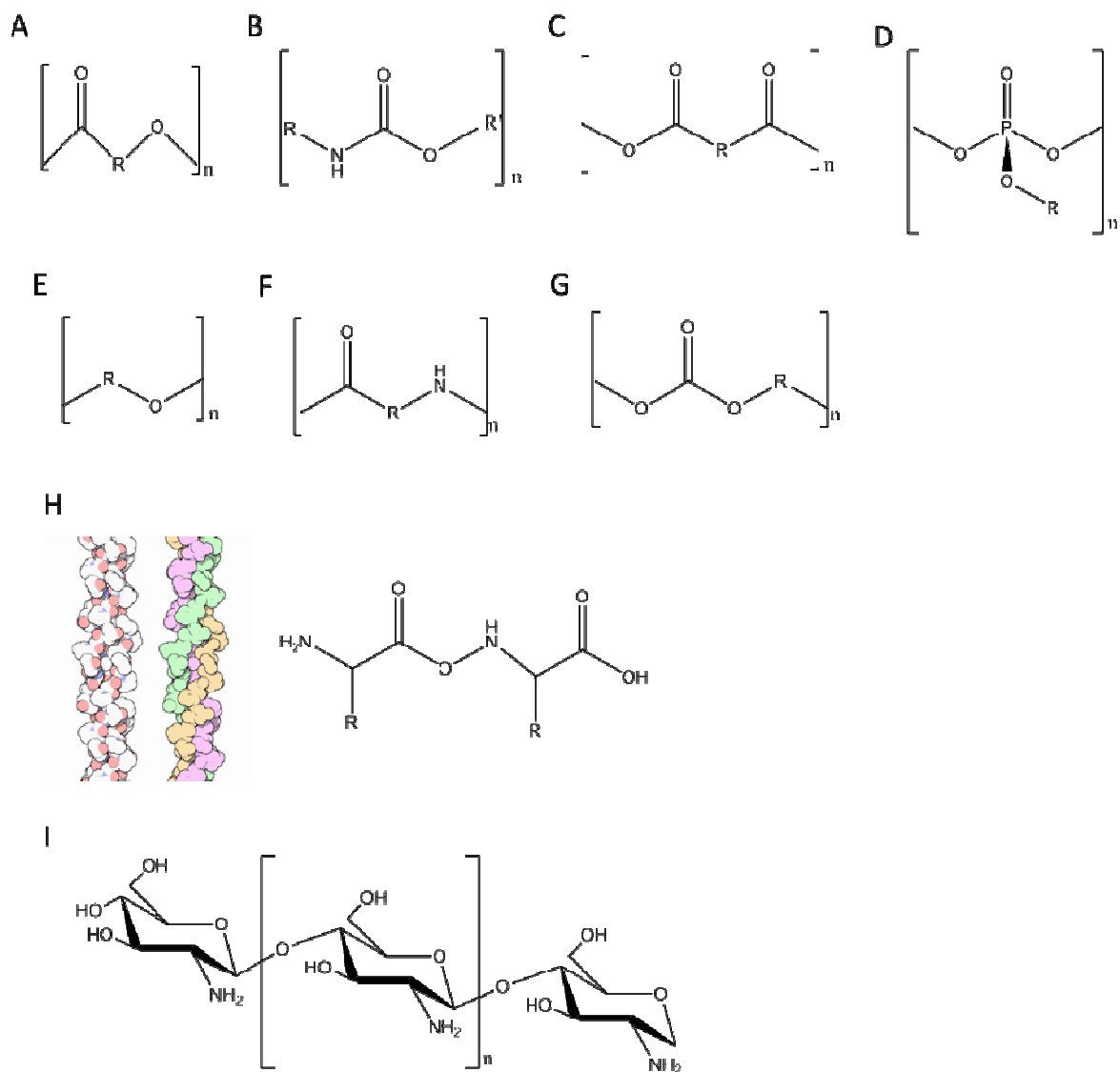
There are several fabrication methods of particles depending on the desired size (microparticles vs. nanoparticles). The most commonly used techniques for microparticle fabrication include hot-melting, emulsion solvent extraction, phase separation, and spray drying or atomization [39]. Methods for nanoparticles fabrication

are classified in two main groups: phase separation and solvent evaporation and chemical reactions including polymerization and polycondensation [15].

Microparticles and nanoparticles are characterized in terms of their morphology, size distribution, and  $\zeta$ -potential by photon correlation spectroscopy (PCS) or dynamic light scattering (DLS). Briefly, a monochromatic light is directed through a suspension of particles in Brownian motion. When the light hits a particle it causes a Doppler shift, changing the wavelength of the incoming light. The change in wavelength is related to the size of the particle [40]. A modified method from PCS is nanoparticle tracking analysis (NTA) by NanoSight Ltd. This technique includes an ultramicroscope and computer software that tracks particles movements and estimates their hydrodynamic radius, using the Stokes-Einstein equation [41]. In addition, electron microscopy techniques including scanning electron microscopy (SEM), transmission electron microscopy (TEM) and surface probe microscopy techniques such as atomic force microscopy (AFM) are commonly used to characterize particle morphology, surface roughness, and size distribution [41]. For SEM analysis, the sample must be a dry powder coated with a conductive metal to visualize the surface of the particles from the secondary electrons emitted. TEM is a technique that is very time consuming during the preparation of the sample, because the samples must be ultrathin and deposited onto grids. AFM is capable of imaging non-conductive material mounted on a sharp probe, so further preparation of the material is not required after mounting on probe [41].

### 2.2.2.2 Applications

Extensive research has been conducted to develop effective delivery systems based on biodegradable polymers for drug delivery and vaccine formulations among other biomedical applications. Hydrolytically degradable polymers that have been used for biomedical applications include polyesters, polyurethanes, polyanhydrides, polyethers, polyphosphoester, polyamides, and polycarbonates. Enzymatically degradable polymers include: proteins and poly(amino acids) (e.g. collagen, elastin, albumin, fibrin) and polysaccharides (e.g. chitosan) [42] (**Figure 2.3**). **Table 2.1** presents an overview of representative applications for some of the most commonly investigated biodegradable polymers. A particular application relevant to the work herein is the use of biodegradable polymer based particles for intranasal vaccine design. The more relevant polymers for this application are discussed in **Section 2.2.3**.



**Figure 2.3 Chemical structures of commonly used polymers for biomedical applications.** Hydrolytically degradable polymers include: polyesters (a), polyurethanes (b), polyanhydrides (c), polyethers (d), polyphosphoester (e), polyamides (f), polycarbonates (g). Enzymatically degradable polymers include: proteins such as collagen (h) (peptide bond structure is presented at the right of a three-dimensional structure of collagen) and polysaccharides such as chitosan (i).

**Table 2.1 Biodegradable polymers and their most representative applications**

<b>Biodegradable polymers</b>	<b>Representative applications</b>	<b>References</b>
<b>Synthetic polymers</b>		
<b>Polyesters</b> <b>Poly(glycolic acid), poly (lactic acid) and copolymers</b>	Drug delivery, vaccine design, tissue engineering, gene delivery and barrier membranes.	[43-48]
<b>Polyanhydrides</b> <b>Poly[1,8-bis(<i>p</i>-carboxyphenoxy)-3,6-dioxaoctane] (CPTEG), poly[1,6-bis(<i>p</i>-carboxyphenoxy) hexane] (CPH), poly(sebacic acid) (SA), poly(fumaric acid) (FA), 1,6-bis(carboxyphenoxy) hexane (MCPH) and copolymers</b>	Drug delivery, vaccine design and orthopedic applications.	[19, 49-52]
<b>Polyethers</b> <b>Poly(ethylene glycol) (PEG) and poly(propylene glycol) (PPG)</b>	As modification on proteins and devices for pharmaceutical industry, drug delivery and gene delivery.	[49, 53-55]
<b>Other synthetic polymers</b>		
<b>Poly(alkylcyanoacrylates)</b>	Adhesives, drug delivery	[56-58]
<b>Polycarbonates</b>	Drug delivery	[59]
<b>Polyamides</b>	Drug delivery	[60]
<b>Polyurethanes</b>	Medical devices	[61]
<b>Natural polymers</b>		
<b>Collagen</b>	Drug delivery, artificial skin, tissue engineering, and coating to improve cellular adhesion or guide tissue regeneration	[45, 62-66]
<b>Fibrogen</b>	Tissue sealant	[67, 68]
<b>Cellulose</b>	Wound healing device, tissue engineering	[69, 70]
<b>Chitosan</b>	Drug delivery, vaccine design, tissue engineering	[71-73]

### **2.2.3 Controlled delivery of intranasal vaccines**

There are just a few vaccine adjuvants currently in the market that are approved for human use. It is evident that a critical need exists for novel immunomodulators and

potentiators, as well as delivery vehicles with the ability to trigger an effective humoral, cellular and mucosal immune response [74]. Recently, the development of potential delivery candidates for intranasal vaccines has gain relevance. Even though most of the currently available vaccines are injected systemically with few exceptions (i.e., oral vaccines against polio and *Salmonella* and the intranasal vaccine against influenza) [75], research on the design of intranasal vaccines has grown enormously. This is because of the ability of mucosal vaccination to induce both local and systemic immunity [76].

A variety of polymeric particles have been considered as vaccine delivery vehicles; however, very few of them have been studied for the design of intranasal vaccines. The most commonly investigated polymers are polyesters, polyanhydrides and chitosan. The size of particulate systems has an important effect on the distribution of particles in the respiratory track after an intranasal administration. Larger particles are trapped in the nasal cavity or upper respiratory track, while smaller particles have the capability to distribute in the lower respiratory track and even reach alveoli [77, 78]. Nanoparticles with a diameter between 100 and 500 nm are most efficient in passing through the nasal cavity into the lungs [79].

Vaccination strategies might vary from forming a depot in the nasal cavity, from where the release of antigen can generate an immune response favoring antigen transport to the nasal-associated lymphoid tissue (NALT) to targeting particles to relevant immune cells like alveolar macrophages located in a deeper location in the respiratory track where the transport to bronchus-associated lymphoid tissue (BALT) is favored [80]. A comparison of the efficacy of both strategies has not been investigated,

and studies have been limited to antibody titer evaluation after administration of microparticles and nanoparticles in which contradicting results are available. Some authors have reported no significant difference between sizes [81, 82] and other reports show a clear effect of particle size [83, 84].

In terms of chemistry, nanoparticles modified with carboxyl, sulfate, or amidine groups have a slow transport across mucosal surfaces; among these groups, the fastest transport was achieved by the most hydrophilic surface (i.e. amidine) [85]. The slow transport across the mucosal surface is a disadvantage, because such particles can be cleared from the mucosal tract [86]. Other studies have shown that particles with high amounts of low molecular weight polyethylene glycol (PEG) on their surface improved the transport of nanoparticles by three orders of magnitudes [87]. Several authors have explained that PEG chains may establish an adhesive interaction due to their ability to inter-diffuse with the mucus network and form hydrogen bonds [86]. As stated before, the effectiveness of these carriers as intranasal vaccine delivery systems depends on the interaction of the particles with the mucosal surface, the recognition and uptake by relevant immune cells (i.e. alveolar macrophages), and the controlled release of the antigen in an active form, with the purpose of eliciting a more potent and long lasting immune response [88].

#### **2.2.3.1 Polyesters**

The chemical structure of polyesters is shown on **Figure 2.3 A**. Polyesters are degradable polymers, which have been approved by the U.S. Food and Drug Administration (FDA) for particular applications in humans (i.e., sutures, vaccine and drug delivery) [89]. Some of the specific polyesters that have been studied for vaccine

delivery applications include poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(3-hydroxybutyric acid) (P3HB), poly(4-hydroxybutyric acid) (P4HB), and poly( $\epsilon$ -caprolactone) (PCL) [90]. Polyesters exhibit biodegradability, controlled release capacity, and biocompatibility [91-93].

In particular, PLGA and PLA have been investigated for their capabilities to be used as intranasal vaccine delivery vehicles. Contradictory results have been reported in the performance of PLA particles eliciting a proper immune response after intranasal administration. Some reports show an enhancement in immunoglobulin G and/or A (IgG and/or IgA) titers after administrations [94, 95], while others report poor performance of PLA particles alone [35, 96, 97]. An improvement in their performance has been reported when used PEG-PLA particles have been used [35, 97]. PEG has the ability to neutralize charge on the particles, increase hydrophilicity, and enhance particle diffusion in mucus [98]. PLGA microparticles have shown a potent induction of systemic and mucosal immunity after intranasal administration [99, 100]. However, their acidic degradation products are a major concern for the stability of antigens [101, 102]. In addition controlling antigen release kinetics is difficult since they erode by bulk erosion mechanism (**Figure 2.2**), which rapidly releases the encapsulated antigen. The ratio of glycolic acid to lactic acid determines the erosion rate, but high levels of glycolic acid increases crystalline domains in the polymer, which reduces its solubility in toxicologically acceptable solvents [103].

### 2.2.3.2 Polyanhydrides

**Figure 2.3 C** shows the chemical structure of polyanhydrides. This class of polymers is hydrolytically degradable and FDA has approved a polyanhydride-based device for



drug delivery to humans [104]. The surface erosion mechanism (**Figure 2.2**) is characteristic of polyanhydrides, which enables well-controlled drug/antigen release kinetics that can be modified by the chemistry of the polymer [105, 106]. In addition, the biocompatibility of the polyanhydrides and their degradation products has been reported [107-109] and a wide range of payloads can be encapsulated into these materials [110].

Polyanhydrides have been used as drug delivery platforms, including vaccine delivery systems [19, 50, 52, 111-113]. Moreover, polyanhydride microparticles and nanoparticles have been shown to be capable of stabilizing proteins and sustain their release [114-116]. As published previously, polyanhydride particles made of copolymers of sebacic acid (SA) and 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) have immunomodulatory behavior *in vivo* [52]. In addition, a novel monomer, 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), has been copolymerized with CPH to engineer amphiphilic environments to improve the stability of encapsulated proteins [116]. In addition, the environment due to the degradation products does not experience drastic changes in pH [117]. Recently, polyanhydrides were found to elicit Th1 responses by acting as agonists for Toll-like receptors (TLRs) 2, 4 and 5 [113].

However, few studies have exploited the use of polyanhydrides as intranasal vaccine delivery vehicles and adjuvants. The 50:50 CPTEG:CPH copolymer has been used successfully in the design of an single-dose intranasal vaccine against *Yersinia pestis* [19]. In addition, some studies suggest that anhydride chemistries enhance the transport of particles on mucosal surfaces. A study shows that PLGA/polyanhydride microspheres enhanced the nasal delivery of a drug when compared with PLGA microspheres alone [118]. Others have shown that polyanhydride nanoparticles (i.e.,

FA:SA copolymer) could be absorbed across rat PP and normal intestinal epithelia [119]. This suggests their potential use as effective vaccine delivery systems, but further studies should be performed, such as the interaction of these polymers with relevant immune cells (as addressed in this thesis) as well as their biodistribution after an intranasal administration.

### **2.2.3.3 Chitosan**

Chitosan (**Figure 2.3 I**) is a polysaccharide that has also shown significant promise as a degradable vaccine delivery vehicle. Its degradation is mediated by papain enzyme and the biocompatibility of the polymer and degradation products has been demonstrated [120-123]. It has been widely used as a nasal delivery vehicle that elicits high levels of IgG and IgA titers [124-128]. However, some studies report that the immune response elicited is more dependent on the route of delivery due to its mucoadhesive properties than its adjuvant properties [90, 129]. For example, intranasal delivery of N-trimethyl chitosan loaded with diphtheria toxoid enhanced the immune response over alum-based vaccines [130]. Nonetheless, Mohanan *et al.* [131] showed a sensitive immune bias in mice when vaccinated through subcutaneous, intradermal, intramuscular, and intralymphatic routes with ovalbumin-loaded N-trimethyl chitosan nanoparticles.

## **2.3 Engineering intranasal vaccines: Immunological aspects**

### **2.3.1 Introduction**

The design of effective vaccines is a complex challenge that has gained importance over the past three decades [132]. Vaccines available in the market have

been developed empirically, with no profound understanding on how they activate the immune system [133]. As stated by Plotkin [134, 135], the efficacy of current vaccines is based on the induction of protective antibodies, or an antibody-mediated immunity. This concept includes components such as high antibody titers or high amount of antibodies, antibody avidity, which refers to the quality or functional characteristics of antibodies, and antibody persistence or generation of memory B cells. However, other elements are crucial for many infectious diseases (e.g., HIV, malaria, tuberculosis, influenza) that require the induction an integrated induction of T cell memory as well as antibody-mediated immunity [132]. The important role of B cells in vaccine efficacy should be integrated to an effective T cell response [136]. The synergy of these two immunities is a complex challenge that is under investigation and has been conceptualized as vaccine-mediated immunity [137]. New studies focus on understanding how adjuvants or antigens are recognized by the immune system and how they prime effector cells and antibodies for more efficient design of protective vaccines [133].

Even though there is growing interest in vaccine development and research, the optimal immune parameters (e.g. cytokine secretion, cell surface marker expression, antibodies titter, CD4 or CD8 T cell propagation) that are associated with an effective vaccine-mediated immunity remain unclear. Some authors have categorized immune responses observed as absolute correlates, relative correlates, co-correlates or subrogates to a vaccine when they provide near 100% protection, provide protection usually, provide protection in a species dependent manner, or does not provide protection, respectively [137]. New methods and techniques have emerged to assess immune parameters and their relationship with vaccine-mediated immunity such as

advances in flow cytometry [138], measure of cytokine production [139, 140], evaluation of T cell-dendritic cell interactions [141], assessment of macrophage phagocytosis and uptake [142, 143], among others.

The development of these new technologies has acquired relevance together with the emergence of new methodologies to determine the safety of effective vaccines [133, 144]. Novel antigens have been proposed in order to overcome safety and efficacy challenges, including recombinant proteins [145-147], purified [148, 149] and synthetic [150, 151] microbial antigens, and DNA and RNA molecules [152-154]. Complementing the urgency to improve the safety and efficacy of vaccines, the discovery and design of vaccine adjuvants has been a key element and novel adjuvants have been investigated [155, 156]. Adjuvants enhance antigen-specific responses when they are co-administered with the antigen to elicit early, more potent, and long-lasting immune response [157, 158]. In addition, adjuvants can be used to reduce the amount of antigen and/or number of immunizations needed to generate a proper immune response, to improve the vaccination efficiency in newborns, elderly or immune-compromised persons, or as delivery systems to target antigen presenting cells (APC) [159]. An “ideal adjuvant” is one that ensures biocompatibility (prevention of toxic effects), that can be manufactured consistently, that elicits a protective immune response with fragile antigens, and that preserves the immunogenicity of the antigen [160]. An additional desirable function is that an adjuvant can serve as immune modulator and controlled delivery vehicle.

The design of effective adjuvants should not be a “mix and match” strategy that combines antigens and adjuvants, but requires a deep understanding of how innate and

adaptive immunities are connected. In this regard, the rational selection of specific adjuvants and specific antigens for particular diseases strongly depend upon the desired immune response. In addition, for the design of intranasal vaccines, the study of lung immunity is essential.

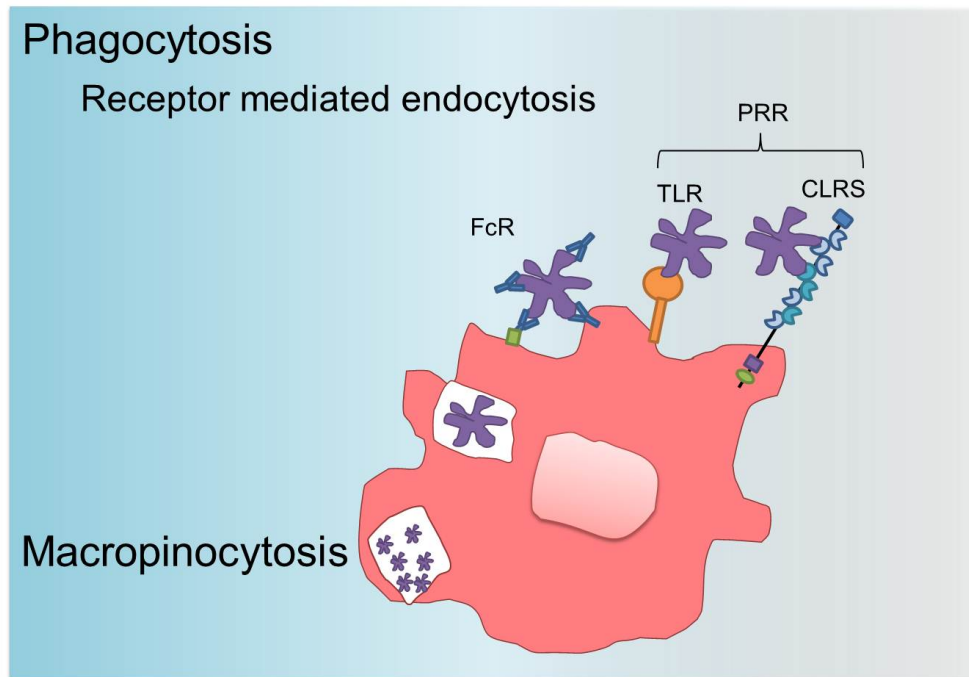
### **2.3.2 From innate to adaptive immune response**

Immune memory is the capability of rapid activation and response of the immune system upon re-exposure of the host to a pathogen. Indeed, this is the very foundation of vaccination [161, 162]. Vaccination is the administration of an antigen to stimulate the immune system to trigger an adaptive immunity to a specific disease and elicit immune memory. An antigen is a particular molecule that binds specifically to an antibody; however an antigen may or may not have the ability to generate an immune response by itself. Particular antigens with this capability are called immunogens [158].

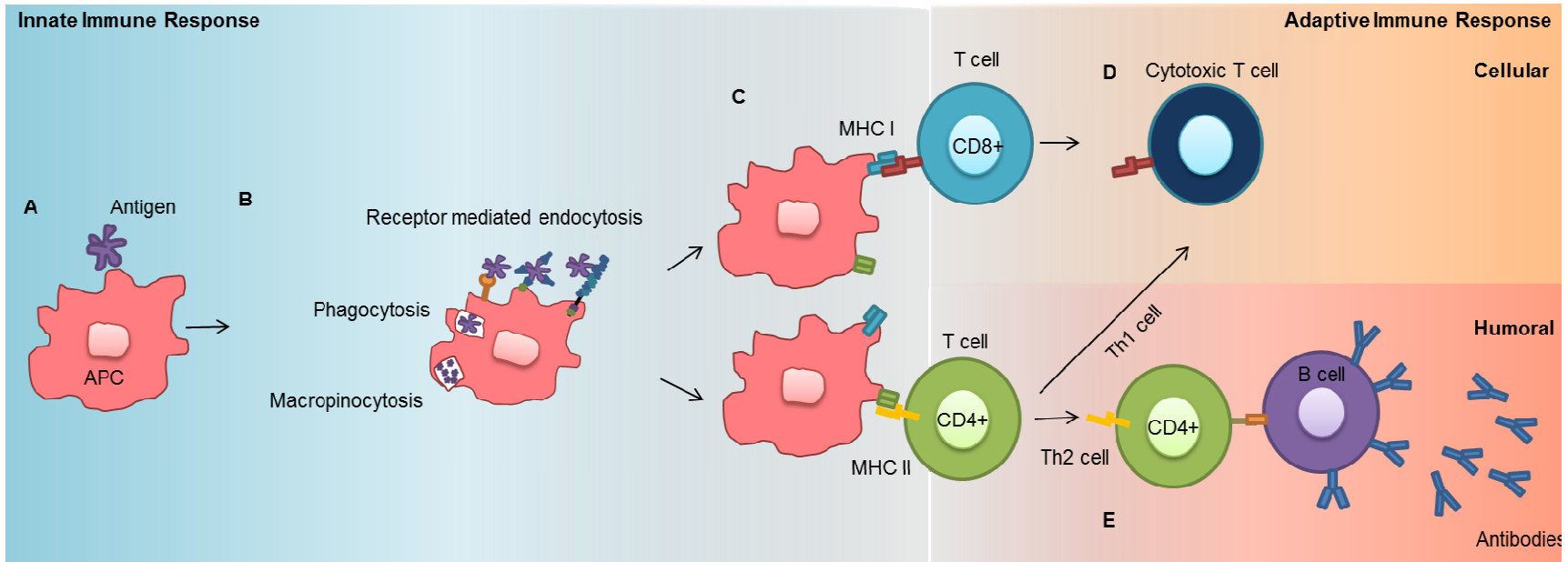
Immunity involves both specific and nonspecific components and can be broadly categorized into adaptive immunity and innate immunity. Adaptive immunity includes T and B lymphocytes its main characteristics are specificity (recognition of very precise antigens) and memory [158]. Innate immunity is considered to be a nonspecific response, with the capability of discriminating between pathogens and self-antigens. It comprises of phagocytic cells among other first-line of defense (e.g. natural killers, granulocytes, complement system) cells [158, 163]. The activation of the innate immune response is needed for the activation of adaptive immunity [163-166].

As explained by Janeway [158], the innate immune system triggers an adaptive response in a series of steps. Briefly, antigen is encountered by APCs and is taken up

and internalized by several mechanisms (**Figure 2.4**), which have been described previously [167-169]. In general, depending on the antigen/pathogen size, chemistry, chemical patterns on its surface, or opsonization of antibodies, it is engulfed and processed either in endosomes or in cytosol. It is also possible that after destabilization of endosome, the antigen is released into the cytosol. Then the antigen is presented on the surface of APCs by major histocompatibility complex class I (MHC I) when processed in the cytosol or by class II (MHC II) when processed in endosome. Cross-presentation pathways can also be activated (both MHC I and MHC II). APCs migrate from the periphery to lymphoid organs, where antigen presented in MHC I activates CD8<sup>+</sup> T cells, and antigen presented in MHC II activates CD4<sup>+</sup> T cells. In order to activate T cells, co-stimulatory signals are needed, such as the expression of co-stimulatory molecules on the surfaces of APCs (i.e. CD40, CD86, CD80), the release of pro-inflammatory cytokines (e.g. IL-1 $\beta$  and TNF- $\alpha$ ), and the production of other cytokines that tailor the immune response (e.g. IL-12, IL-10, and IL-17). Subsequently, CD8<sup>+</sup> T cells can become cytotoxic T cells (CTLs) and CD4<sup>+</sup> cells become T helper cells 1 or 2 (Th1 or Th2) depending on the cytokine signals provided by the APC. CTLs kill virally infected cells and tumor cells, Th1 assists in the activation of CTLs, and Th2 assists B cells so that antibodies can be produced against the particular antigen (**Figure 2.5**).



**Figure 2.4 Uptake of antigens by antigen presenting cells.** There are several endocytosis mechanisms that APCs use to uptake and internalize antigens/pathogens [167-169]. Phagocytosis (engulfment of large entities) includes receptor mediated endocytosis that comprises the internalization upon recognition through pattern recognition receptors (PRRs) (i.e. toll like receptors or TLRs, and C-type lectin receptors or CLRS). Macropinocytosis refers to the engulfment of small entities.



**Figure 2.5 Innate and adaptive immune response.** The innate immune system triggers an adaptive response by encountering an antigen (A), which it is taken up and internalized by several mechanisms (B). After processing, the antigen is presented on the surface of APCs (C) to T cells including CD4<sup>+</sup> and CD8<sup>+</sup>. After antigen recognition, CD8<sup>+</sup> T cells can become cytotoxic T cells (CTLs) (D), while CD4<sup>+</sup> cells become T helper cells 1 or 2 (Th1 or Th2) (E).



### 2.3.3 Lung immunity

Two aspects drive the progress of “pathogen-mimicking” strategies: drug formulation and route of administration; both are key elements for the efficient design of vaccines. In particular, for vaccines against respiratory pathogens, intranasal administration mimics the entry of pathogens into the organism. It has been published that intranasal immunization has provided local and systemic robust immune responses against respiratory pathogens [170-174], which makes it an attractive route for the development of new vaccines. In particular, for the design of respiratory vaccines, the transport of particles across mucosal surfaces is crucial [175] and particle size and chemistry are important characteristics to improve transport efficiency [79, 85-87]. Pathogens can be mimicked by incorporating elements to the vaccine formulation that will lead the immune system sense danger signals similar to the actual infection [176]. A profound understanding of the respiratory tract immunity is needed so that a rational strategy can be formulated for the efficient design of vaccines.

The respiratory tract is usually classified into two main parts, the upper and the lower zones. The upper respiratory tract comprises the nasal cavity, pharynx, and larynx. The lower respiratory tract includes trachea, primary bronchi, and lungs [177]. The protection of lungs is associated with a series of anatomical barriers that vary through the tract, including mucociliary apparatus, enzymes, secretory IgA, and phagocytic cells that are constantly sensing the environment in order to detect the presence of pathogen [178]. After pathogen recognition, it is cleared and/or transported to draining lymph nodes [178].

The mucosa-associated lymphoid tissues (MALT) are dispersed aggregates of organized lymphoid tissue within the mucosa; which are responsible of mucosal-surface immune responses [179]. MALT includes pulmonary, nasal and gut associated lymphoid-tissue and together are known as the largest mammalian lymphoid organ system [180]. The bronchus-associated lymphoid tissue (BALT) and the nasal-associated lymphoid tissue (NALT) are capable of generating T and B cell responses to inhaled antigens. BALT is located at bifurcations of the bronchus and it is present since the fetus development, and it matures after birth. NALT is located in the nasal passage and develops after birth [80]. Isotype switching of naive B cells to IgA and differentiation of T cells to Cytotoxic T cells (CTL) has been demonstrated on both BALT and NALT [181, 182], confirming that both cellular and humoral immunity can be triggered from this lymphoid tissues. Naïve B and T cells arrive to MALT via high endothelial venules [183, 184]. Then, antigens are presented to the naïve T cells by APCs after intracellular processing. Furthermore, luminal antigens may be taken up and presented by B lymphocytes and epithelial cells to subsets of T lymphocytes [183]. Subsequently, naïve B cells are activated to become effector or memory cells. It is common that isotype switching occurs directly or sequentially (via IgG) to IgA. In fact, MALT structures are remarkable generators of IgA and J-chain expression (needed for its secretion) [158, 184].

Novel adjuvants have been used to target APCs from which an innate immune response can be initiated, subsequently eliciting an adaptive response. Much research has focused on the activation of DCs in order to elicit an immune response. However, depending on the type of vaccine, other APCs might play a principal role in eliciting

robust immune responses. In the respiratory tract, especially in the alveoli in the deep pulmonary region, alveolar macrophages (AM $\phi$ ) have special relevance as the first line of immune defense. An interesting finding, relevant to the design of intranasal vaccines, is described by Kirby *et al.* [185] in which it was observed that >80% of cells obtained from bronchoalveolar lavage (BAL) were identified as AM $\phi$ . These studies showed that AM $\phi$  not only have a greater probability to encounter pathogens in the respiratory tract, but also that AM $\phi$  were the first cells to transport pathogen to the lung draining lymph nodes [185]. This suggests that AM $\phi$  are in charge of the connection between the innate and the adaptive immune response in lung immunity transporting antigens to the BALT and its activation is relevant when vaccines are design to target the lower respiratory tract.

#### **2.3.4 Macrophages**

Macrophages (M $\phi$ ) are white blood cells produced by the differentiation of monocytes in tissues [158]. According to Mosser and Edwards, there is a full spectrum of M $\phi$  that share some characteristics, but actually play different functions going from classically activated, regulatory, to wound healing [186]. In particular, Hume [187] has stated that classical activated M $\phi$  perform similar functions as DCs and even have a greater potential to present antigen to T cells due to their higher propensity to take up antigen. The APCs described as “professional” include macrophages, Langerhans cells, and dendritic cells (i.e. myeloid and lymphoid DCs), but B cells can also perform professional presentation of antigens [188, 189].

An abundant population of M $\phi$  is contained in most tissues (up to 15% of total cells) [190-192], which generates several phenotypes depending on the tissue. The plasticity of these cells is remarkable. In addition, its phagocytic activity is extremely high even in the absence of inflammatory signals. The process of phagocytosis *per se* enables the presentation of the antigens; their exposure to specific cytokines and danger signals up-regulates the expression of co-stimulatory signals that contribute to prime and amplify the response of T cells [193]. In addition, other functions are conferred to M $\phi$ , such as chemotaxis, phagocytosis and pathogen killing, clearance of dying and dead cells, tumor-cell control, and secretion of immune modulators and enzymes (e.g. nitric oxide, proteases, cytokines, reactive oxygen intermediates, coagulation factors, etc.) [193].

Alveolar macrophages (AM $\phi$ ) are the major defense cells in the lung playing an important role in inflammation, control of infection and antigen transport to draining lymph nodes [185, 194, 195]. In addition, their important role as regulatory cells, managing the homeostasis of the lung, is fundamental. AM $\phi$  have the capability to direct suppressive signals [196, 197]. This implies that the rational design of intranasal vaccines has to take in consideration the homeostatic role of AM $\phi$ , so that vaccine-induced signals can elicit a potent immune response and not tolerance. AM $\phi$  are equipped with receptors to recognize foreign, altered-self, and self-components [198]. In fact, AM $\phi$  recognize danger invaders by recognizing pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs). Toll-like receptors (TLRs) and C-type lectin (CLRs) are important PRRs that recognize widely distributed structures in pathogens. Particularly, CLRs recognize carbohydrate structures, which

are extensively expressed on the surface of respiratory pathogens. CLRs are considered a key component of the immunity in lungs [199].

## **2.4 Targeted vaccine design**

### **2.4.1 Introduction**

Efficient vaccines should be engineered to mimic an infection, in order to provide protective immunity to disease, but without the undesirable effects of the pathogen. A pathogen can be mimicked by non-infectious materials in several aspects; for example, using the route that the pathogen uses to infect the host, and providing elements that can be recognized by immune cells as foreign [200, 201]. The use of biodegradable particles as adjuvant/delivery vehicles can be enhanced by targeting strategies, which can improve the delivery of the payload to particular tissues or cells and overcome biological barriers, as well as provide appropriate danger signals to elicit a robust immune response.

Targeting strategies can be classified in two broad groups: passive and active [202-204]. Passive targeting strategies are focused on changing particle size and chemistry to achieve higher delivery efficiency. Active strategies are oriented to target specific cells or tissues, using ligands on the surface of particles that bind to specific receptors, or antibodies for a higher specificity in the binding of the receptor. The latter strategy will be discussed in this section, since passive targeting strategy was discussed in **Section 2.2**.

It is important to highlight that appropriate targeting depends on the specific application of the vaccine. Although vaccines have been understood as prophylactic strategies, the development of novel vaccines has changed this concept. Currently,

therapeutic vaccines have been investigated as a method to activate the immune system against illnesses such as cancer and HIV/AIDS [205-207]. In particular, the targeting of M $\phi$  plays an important role in the development of therapeutic vaccines, because of their ability to inhibit replication of intracellular parasites, tumor growth, and induction of an immune response [208]. However, the aim of this section is to illustrate immune targets and targeting strategies for the specific purpose of designing a prophylactic vaccine against pathogens.

#### **2.4.2 Antigen presenting cells as immune targets**

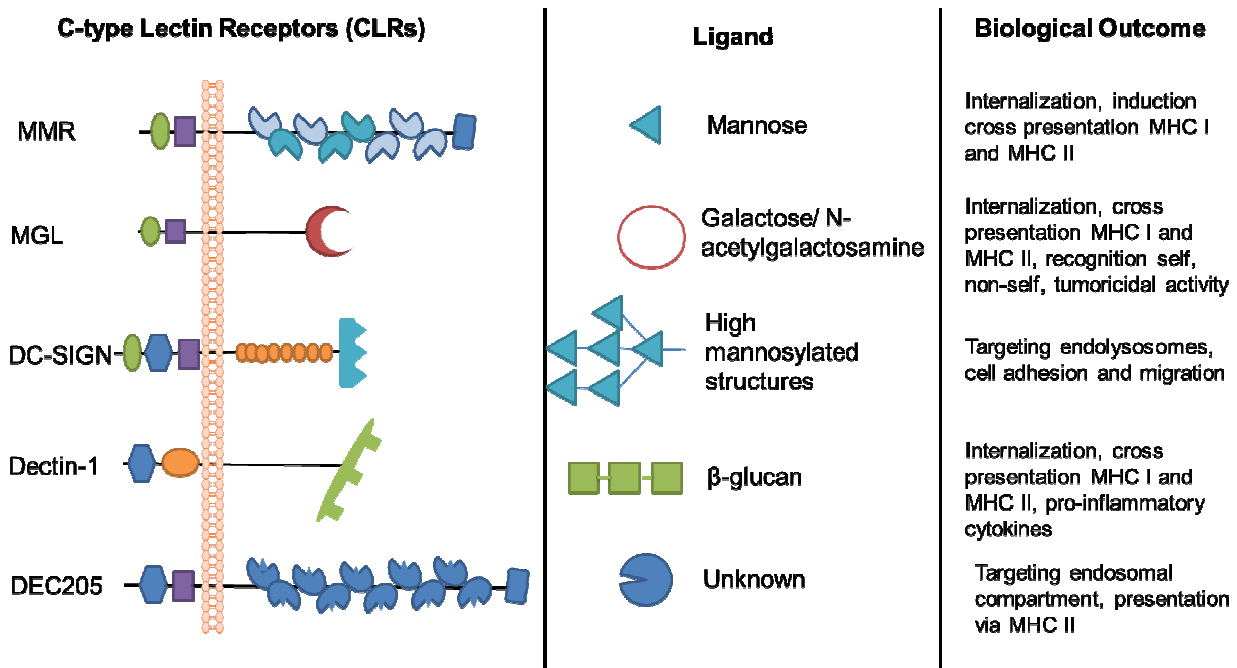
Targeting specific receptors is an interesting strategy in the rational design of novel adjuvants. It is an effective strategy to enhance uptake and internalization of antigenic or genetic materials by APCs. In fact, a wide distribution of PRRs could be immune targets with the potential to tailor the immune response. PRRs include Toll-like receptors (TLRs), NOD-like receptors, RIG-I-Like Receptors (RLRs), and C-type lectin receptors (CLRs) [209-211].

Vaccination strategies include the modification of antigen as well as functionalization of surface particles, both employing targeting agents. The potential of functionalized polymer particles with specific ligands that can be recognized by PRRs is enormous for the development of novel adjuvants. Several researchers agree that targeting PRRs allows a more specific, efficient and less toxic response [165, 166, 212-218] that has the capability to “shape” the immune response [164, 165, 209, 210].

#### 2.4.2.1 C-type Lectin Receptors

CLRs are excellent targets when the purpose is to trigger the innate response because of their high expression by APCs and endocytic properties. The regulatory role of the innate response and the triggering of adaptive immune responses through the effector function of APCs usually requires the collaboration of other PRRs, i.e. TLRs [219]. CLRs perform various functions, such as cell adhesion/migration, tissue integration, pathogen recognition and internalization (i.e., endocytosis, phagocytosis), cell activation and immune induction and modulation, etc. [220]. It is important to state that depending on the binding properties of the antibody or ligands, immune response, tolerance or suppression can be generated [216, 218, 221].

Despite the existence of a wide range of CLRs with similar or overlapping ligand specificity, each receptor appears to have distinct functions depending on the context in which the ligand is recognized [222]. **Figure 2.6** shows some of them with their respective ligands and function.



**Figure 2.6 C-type lectin receptors.** CLRs recognize a wide variety of carbohydrates; the most representative as well as the known biological outcome after cross-linking with the receptor are presented in this figure. In particular, DEC205 has several carbohydrate recognition domains, but has been targeted using antibodies, since its carbohydrate ligand remains unknown.

#### 2.4.2.2 Toll-Like Receptors

Toll-Like Receptors (TLRs) recognize different molecules ranging from lipopolysaccharides to nucleic acids, and the synergy between them is singular [158]. Three of the most important TLRs are TLR 2, TLR 4 and TLR 9 because of their function in modulating the immune response [165, 223]. TLR2 recognizes lipoproteins, glycolipids and lipopolysaccharides. Its activity is determined by the interaction with other TLR such as TLR6 (recognize lipopeptides and zymosan particles) and TLR1 (its main ligand is lipoprotein). TLR2 by itself does not induce cytokine production, but it does after the interaction with activated TLR6 or TLR1 [165].



TLR4 recognizes lipopolysaccharide (LPS) which is an integral component of the outer membrane of gram negative bacteria [158]. In humans, TLR4 is not activated upon the only recognition of LPS, suggesting that an additional molecule is required for downstream signaling [165]. In fact, it is known that TLR4 is a signaling receptor, but not the LPS uptake receptor. The LPS uptake mechanism is still unknown [223].

The recognition of bacterial DNA is attributed to TLR 9 [158]. Certain bacterial oligonucleotides that contain unmethylated nucleotides can stimulate APCs because of their recognition through TLR9. However, eukaryotic DNA and methylated oligonucleotides are not recognized by this receptor. After recognition of the ligand, the receptor is stimulated and cells express co-stimulatory molecules, increase antigen presentation, and produce Th1 signature cytokines (i.e. IL-12 and IL-18). The strong Th1 response induced suggests that it is a promising target in the design of adjuvants for vaccines against a wide variety of infectious agents and cancer antigens [165]. Characterization of the TLR signaling pathways is a strategy that leads to the comprehension of the mechanism that links recognition of pathogens and the resultant immune response.

#### **2.4.3 Targeting strategies**

Numerous strategies have been studied for the active targeting of receptors with an eye towards generation of potent immune responses. Strategies include, but are not limited to, antibody-mediated targeting and the direct modification of antigens and/or particles with specific ligands.

#### **2.4.3.1 Antibody-mediated targeting**

Antibody-targeted vaccines take advantage of the high affinity of antibodies to recognize specific molecules. This strategy has been used for the treatment of cancer, using monoclonal antibodies that recognize specific tumor antigens, in order to direct the delivery of chemotherapy drugs and tumor-growth inhibitors [224, 225]. These monoclonal antibodies recognize specific cytokines and molecules that promote angiogenesis and tumor growth, with the purpose to inhibit them [226, 227]. This is an example of the design of therapeutic vaccines in which the activation of immune response is directed to cancer cells. In addition, antibodies have been used to target receptors to which a specific ligand remains unknown (e.g. DEC205) [228, 229]. Moreover, monoclonal antibodies have been used to target specific markers of immune cells in order to direct cargo. Particular receptors of cells, such as CD11b or CD11c, have been used to target particular APCs, resulting in a high antibody titer response, suggesting a successful delivery to APCs [230, 231].

#### **2.4.3.2 Functionalization of antigens and particles**

This strategy might include the use of antibodies [228, 232] and small molecules (e.g. saccharides, amino acids) [233, 234] chemically bonded to antigens or particles with the aim to direct its delivery to immune cells, or provide a directed response depending on the ligand. The applications of these strategies include the activation of the immune response against cancer cells or foreign pathogens. Specially, antigens that have been modified with targeted agents are still challenged in terms of their immunogenic properties and the incorporation of an adjuvant is needed. The use of

polymeric devices not only as delivery vehicles, but also as immunomodulators has helped overcome this problem. The functionalization of the surface of these delivery vehicles provides the opportunity to exploit active targeting strategies. The approach includes the conjugation of different molecules, including antibodies, peptides, and carbohydrates, low-molecular weight polymers, etc. that confer the capability to target specific cells, receptors or tissues, or modification of surface chemistry to improve particle interaction with biological environment. This allows the delivery of the payload in a directed manner [235-239]. Results show the outcome of the strategy depends on the particular immune target and the strategy used, and its rationale depends on the specific application. For example, Copland *et al.* [240] showed improved internalization of mannosylated liposomes by DCs. Another strategy is the linkage of synthetic peptides to the surface polymeric particles. Studies show the *in vitro* efficacy of nanoparticles targeting neurons using tetanus toxin C fragment (TTC) as ligand [241, 242]. The strategies herein presented are an exciting opportunity to improve the design of targeted vaccine platforms.

## 2.5 Conclusions

Biodegradable polymeric particles are promising carriers of poorly immunogenic antigens (i.e. recombinant proteins, nucleic acids, etc.) that preserve their stability and antigenicity. In particular, polyanhydride nanoparticles offer unique characteristics for the design of vaccines including controlled release of the antigen, biocompatibility, protein stability, and immunomodulatory properties. Previous studies have shown the potential of polyanhydride nanoparticles to be used as carriers for intranasal vaccines.

The use of nanoparticles (100-500 nm in diameter) as vaccine carriers is an interesting strategy because these particles can be distributed throughout the respiratory tract, including alveolar regions. AMs are one of the most important immune cells in the respiratory tract and play a key role in translating an innate stimulation to a long lasting adaptive response, transporting antigens to BALT. AMs are armed with PRRs, which sense pathogens and initiate signaling pathways to trigger an immune response. In particular, C-type lectin receptors are predominant elements of lung immunity and targeting CLRs may be a viable strategy for the design of efficacious intranasal vaccines.

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## CHAPTER 3: RESEARCH OBJECTIVES

### 3.1 Overall Research Goal

The overall goal of this project is to design a targeted nanovaccine platform by functionalizing the surface of amphiphilic polyanhydride nanoparticles to target specific C-type lectin receptors (CLRs). In this work, we have evaluated the capacity of these targeted nanoparticles to get internalized by and activate alveolar macrophages, and through mechanistic studies with knockout mice, we have shown that the specific engagement of CLRs plays an important role in their recognition. The results will provide valuable insights in the development of intranasal vaccine formulations. This study focuses on amphiphilic polyanhydrides based on 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis(*p*-carboxyphenoxy)hexane (CPH). The saccharides used to modify the surface of CPTEG:CPH nanoparticles are di-mannose and galactose.

### 3.2 Specific Goals

The specific goals of the project are:

*Specific goal 1:* High throughput synthesis and characterization of functionalized polyanhydride nanoparticles.

*Specific goal 2:* Design of functionalized polyanhydride nanoparticles to target C-type lectin receptors on alveolar macrophages.

## **CHAPTER 4: HIGH THROUGHPUT SYNTHESIS AND CARBOHYDRATE MODIFICATION OF POLYANHYDRIDE NANOPARTICLES**

### **4.1 Abstract**

The rational design of vaccine carriers has integrated several characteristics such as a rational selection of polymer, chemistry, incorporation of various antigens and targeting agents. High throughput fabrication and modification of biomaterials is a powerful tool that increases the ability to study a wide array of devices. In addition, automated set ups increase the reproducibility of the procedures. LabView<sup>®</sup> platform was used to operate a series of three pumps and five different actuators that allowed the automated high throughput synthesis of polyanhydride copolymers, nanoparticle fabrication, and surface modification by attaching carbohydrates. This last procedure was optimized by varying the temperature and the total reaction time. Optimal conditions permitted the efficient surface functionalization of nanoparticles, maintaining their morphology and a consistent carbohydrate concentration on their surface.

## 4.2 Introduction

The use of computational software platforms to operate laboratory equipment is a powerful tool to improve the accuracy and throughput of diverse processes. LabVIEW® (National Instruments, Columbus, OH) is a visual programming tool that can manage various pieces of equipment and perform calculations for a more effective research activities [1]. Its standardized use allows for different research laboratories to replicate published procedures by reducing troubleshooting and building up knowledge. Automated high-throughput strategies for the fabrication or modification of biomaterials enhance the feasibility of constructing consistent and reproducible devices that integrate several desirable characteristics.

The integration of different features on delivery carriers is acquiring renewed importance in the rational design of biomaterials for numerous applications. The variation of parameters such as chemistry [2-5], erosion mechanisms [5-8], size or geometry to target specific cells [9-12], and the incorporation of targeting agents [13-15] has been studied. Increasing the throughput in the fabrication method to unearth the complexities associated with such a multi-parametric system will be of great value.

In vaccine design, polyanhydride nanoparticles offer controlled release of the payload (i.e., antigen) that can be tailored by changing the chemistry of the polymer. Due their hydrophobic chemistry, these polymers experience surface erosion through hydrolytic degradation. By modifying the hydrophobicity of the polymer via copolymerization with different anhydride monomers, controlled release can be achieved, allowing for tailored degradation ranging from days to months [16-18]. In addition, the stabilization of novel antigens has been achieved by introducing

amphiphilic chemistries [8, 16, 17, 19]. Finally, these materials exhibit adjuvant and immunomodulatory properties [20], making them promising candidates as vaccine adjuvants.

The addition of a targeting ligand capable of being recognized by specific receptors on immune cells has been studied [21] to selectively trigger an immune response. C-type lectin receptors (CLRs) are pattern recognition receptors (PRRs) that recognize carbohydrates present on the surface of pathogens. The stimulation of immune cells via CLRs allows for the internalization of antigen and subsequent presentation for further T cell activation; in addition, tyrosine motifs located on their transmembrane tails permits downstream signaling [22-25].

In this work, the development of a high throughput procedure for functionalization of polyanhydride nanoparticles employing an automated robotic set up operated by LabView<sup>®</sup> is described. Temperature and reaction time were the parameters employed to optimize the process, to achieve consistent nanoparticle morphology with a reproducible carbohydrate concentration on the nanoparticle surface.

## **4.3 Materials and Methods**

### **4.3.1 Materials**

The chemicals needed for monomer synthesis, polymerization and nanoparticle fabrication include 1,6-dibromohexane, triethylene glycol, 4-*p*-hydroxybenzoic acid, and 1-methyl-2-pyrrolidinone; these were purchased from Sigma–Aldrich (St. Louis, MO); 4-*p*-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); toluene, sulfuric acid, acetonitrile, dimethyl formamide, acetic anhydride, methylene chloride, pentane,

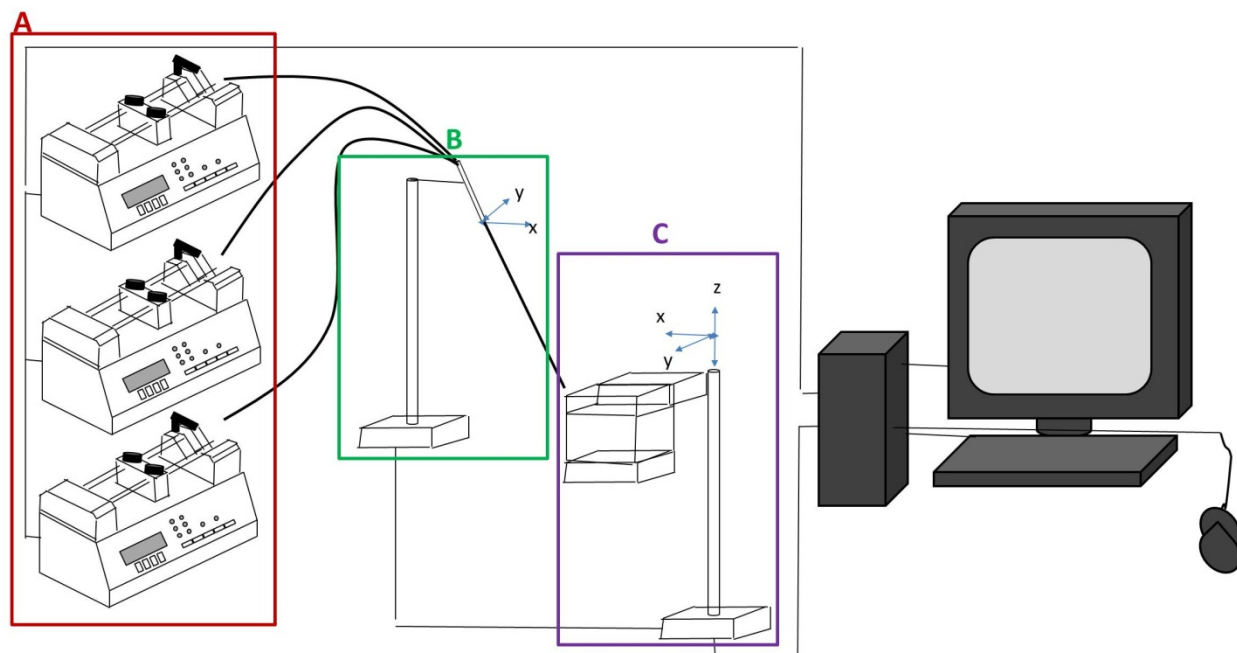
and potassium carbonate were obtained from Fisher Scientific (Fairlawn, NJ); *p*-carboxy benzoic acid (99+%), and 1-methyl-2- pyrrolidinone, anhydrous (99+%), were purchased from Aldrich (Milwaukee, WI). For <sup>1</sup>H NMR characterization deuterated chemicals including chloroform and dimethyl sulfoxide were purchased from Cambridge Isotope Laboratories (Andover, MA).

#### **4.3.2 Monomer and high throughput polymer synthesis**

The 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) and 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) monomers were synthesized as previously described [26, 27]. Using a modified version of the automated robotic apparatus (**Figure 4.1**) described previously [3, 4, 8], 50:50 CPTEG:CPH copolymer was synthesized. Briefly, two single-syringe pumps NE 1000 (New Era Pump Systems, Farmingdale, NY) were connected in series; each pump contained a 10 mL glass syringe (Hamilton, Reno, NV) loaded with each pre-polymer dissolved in acetic anhydride (15 mg/mL) and connected to 24 in. stainless steel capillaries. Pumps were used to infuse desired amount of prepolymers into glass cuvettes. Tips were held on a clamp mounted on a robotic stage with movement in the x and y direction. Glass cuvettes were placed on a metal rack mounted on a robotic stage with movement in the x, y and z directions. Robotic stages were built using automated actuators (Zaber Technologies, Vancouver, British Columbia, Canada) connected in series, one for each direction. Pumps and actuators were connected to a computer and the robotic apparatus was operated using LabVIEW<sup>®</sup>. Prepolymer at the appropriate concentration was polymerized at 0.3 torr vacuum and 140°C. Copolymer films were obtained after polymerization. Discrete libraries ranging from homopolymers (CPH or CPTEG) to copolymers of various



compositions were synthesized using this setup of the deposition apparatus and compared to results from conventional syntheses [3]. The current studies are focused on a single chemistry (i.e., 50:50 CPTEG:CPH).



**Figure 4.1 Schematic representations of robotic deposition apparatus.** The automated deposition apparatus consists of (A) three NE 1000 pumps; (B) a robotic stage integrated by two actuators (Zaber) one for movement in the x direction and another one for movement in the y direction; (C) a second robotic stage with two adjacent racks (appropriate for tubes and cuvettes) consisting of three actuators one for each direction (x, y, and z). The pumps were and a total of five actuators were connected in series. Actuators and pumps were operated by a computer using LabVIEW® software. This diagram is not to scale.

### 4.3.3 Nanoparticle Fabrication

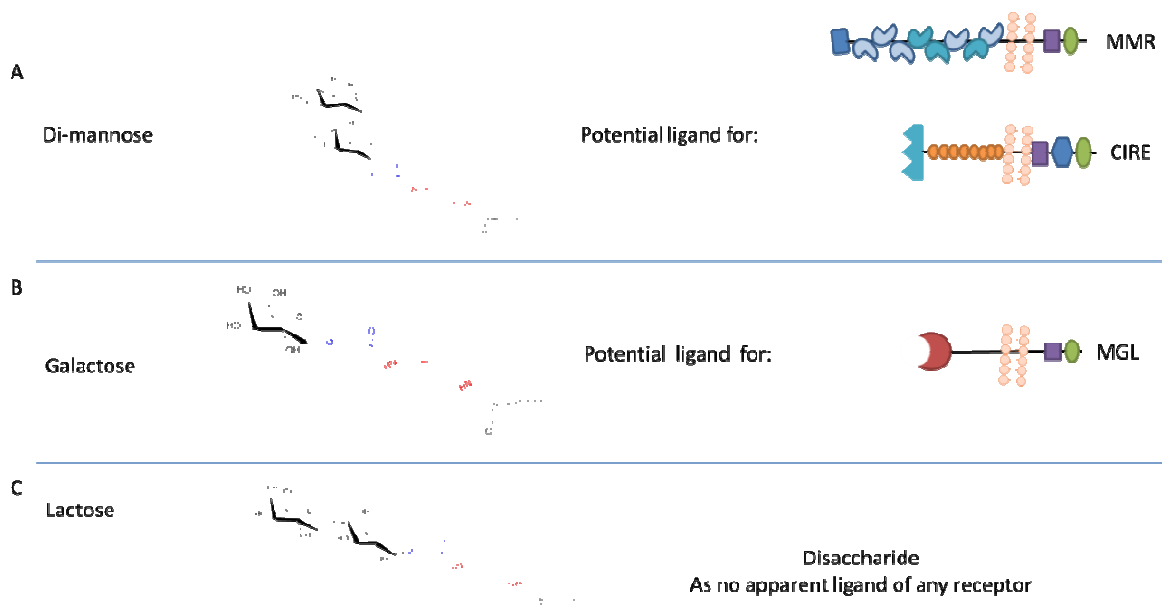
A nanoprecipitation technique was adapted and used from previous work [11]. The precipitation of nanoparticles into an anti-solvent was performed utilizing the automated robotic set up shown in **Figure 4.1**. First, cold methylene chloride ( $-20^{\circ}\text{C}$ ) was loaded into a glass syringe and deposited into glass cuvettes to dissolve 50:50

CPH:CPTEG copolymer at a concentration of 25 mg/mL using one of the pumps. A second pump was used to deposit cold pentane (-30°C) into glass test tubes, held on an adjacent rack mounted on the stage with movement in the x, y, and z directions. A third pump was used to withdraw the dissolved polymer from the cuvettes (at 4 mL/min) and dispensed into test tubes containing pentane (at 7.5 mL/min). Instant precipitation of nanoparticles was observed. The ratio of methylene chloride:pentane was optimized at 1:200. Nanoparticles were filtered or centrifuged (depending on the batch size) to remove pentane and dried overnight in a vacuum chamber.

#### **4.3.4 Surface functionalization**

The rationale for the selection of each specific ligand is shown in **Figure 4.2**. Deprotected saccharides were synthesized by Dr. Nicola Pohl's group in the Chemistry Department at ISU. Deprotected lactose, di-mannose, or galactose were conjugated on the surface of polyanhydride nanoparticles by an amine-carboxylic acid coupling reaction [21], which was optimized by varying reaction times and temperatures. Briefly, the process consisted of two consecutive reactions. For the first reaction, one pump was loaded with a gas-tight glass syringe containing 12 equivalents (eq.) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and 10 eq. of ethylene diamine per 100 mg of nanoparticles in an aqueous solution. A second pump was loaded with a syringe containing 12 eq. of *N*-hydroxysuccinimide (NHS) per 100 mg of nanoparticles in aqueous solution. A nanoparticle suspension was obtained after deposition and incubated at two different temperatures (4°C and 25° C) at a constant agitation using a rotoshaker (Scientific Industries, Bohemia, NY). Nanoparticles were washed twice by centrifuging (10,000 rpm for 5 min) and adding nanopure water and sonicating at 40 Hz

for 30 s. For the second reaction, two deposition steps were used. In the first deposition step, 12 eq. of EDC were loaded with one pump, 12 eq. of NHS with the second pump per 100 mg of particles. A third pump was employed for 10 eq. of glycolic acid (used for the control groups, since deprotected saccharides already have this molecule covalently linked to the saccharide). The second deposition step included 10 eq. of each saccharide on each pump (i.e., galactose, lactose or di-mannose). Each saccharide was deposited into test tubes depending on the desired group. The nanoparticle suspension was incubated at 4°C and 25°C with constant agitation. The times of both reactions were varied as shown in **Table 4.1**. Preliminary studies showed that same temperature was optimal for both reactions and that the second reaction was more responsive to changes in time (data not shown). The nanoparticles were washed once as described previously and dried under vacuum for 6 hours.



**Figure 4.2 Rational design of functionalized nanoparticles.** Three saccharide groups were attached to the surface of 50:50 CPTEG:CPH nanoparticles. In the chemical structures shown, carboxylic acids present on the surface of polyanhydrides and the ethylene diamine group that serves as a linker are represented; (A) shows the chemical structures of the deprotected di-mannose functionalized nanoparticles, which were designed as a potential ligand for the macrophage mannose receptor (MMR), which recognizes mannose residues and CIRE which recognizes highly mannosylated structures. (B) shows galactose functionalized nanoparticles, which were designed as a potential ligand for the macrophage galactose lectin (MGL) receptor that recognizes galactose residues as well as N-acetyl galactosamine. (C) shows lactose functionalized nanoparticles, which do not serve as a specific ligand for any particular receptor.

**Table 4.1 Reaction times used to optimize amine-carboxylic acid coupling reaction**

Total reaction time (h)	First reaction time (h)	Second reaction time (h)
4	2	2
6.5	2	4.5
9	4.5	4.5
13.5	4.5	9
18	9	9
21	9	12
24	12	12

#### 4.3.5 Polymer and nanoparticle characterization

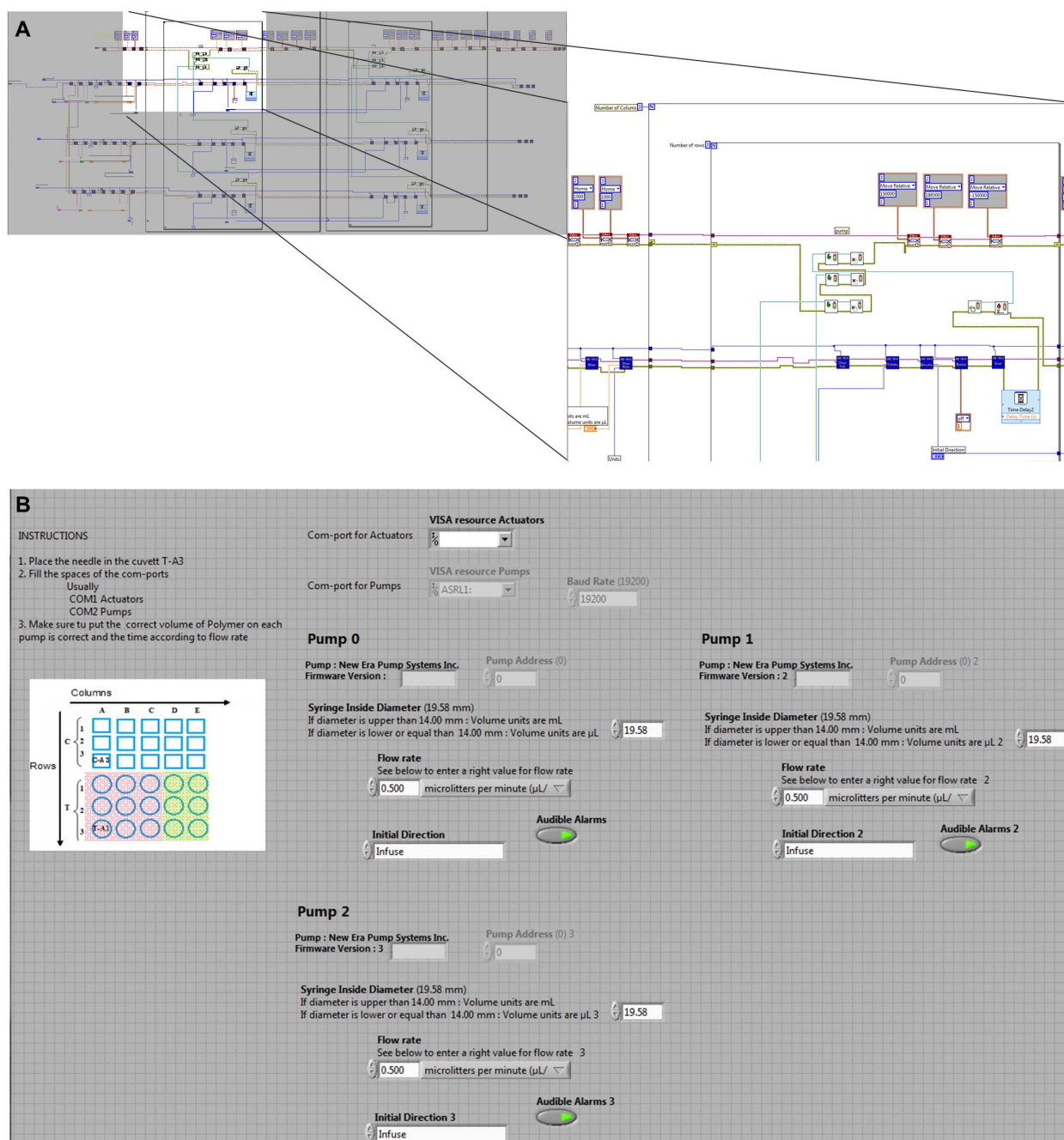
The chemical structure of the copolymer was characterized with  $^1\text{H}$  NMR with a Varian VXR 300 MHz spectrometer (Varian Inc., Palo Alto, CA). Deuterated chloroform was used to dissolve the polymer and the spectra were calibrated with respect to the chloroform peak ( $\delta=7.26$  ppm). The molecular weight was determined using gel permeation chromatography (GPC) using a Waters GPC chromatograph (Milford, MA) containing PL gel columns (Polymer Laboratories, Amherst, MA) and elution times were compared to monodisperse polystyrene standards (Fluka, Milwaukee, WI). Particles were characterized by scanning electron microscopy (SEM, FEI Quanta 250) and dynamic light scattering (DLS, Zetasizer Nano, Malvern Instruments Ltd., Worcester, UK) evaluating their morphology, size and  $\zeta$ -potential. A high throughput phenol sulfuric acid assay previously described [21, 28] was used to quantify saccharide concentration on nanoparticles surface.

### 4.4 Results and Discussion

#### 4.4.1 Translation of operational software

Previous work from our laboratories [3, 8, 9, 29-31] described a similar robotic set up as the one shown in **Figure 4.1**. In that work, a fixed stage and a mobile stage in x, y, and z directions was operated by a third party software that integrated the information from the pumps and actuators. The addition of a second movable stage increased the throughput and reduced the time for each process (i.e., prepolymer deposition and nanoparticle precipitation). The translation of the operation of stages and pumps to LabVIEW<sup>®</sup> improved the ease of use of the set up as well as the

reproducibility of the process, since each process was programmed in a standard manner so that the user can change parameters as flow rate, batch size, concentration, etc. without changing the program code. As shown in **Figure 4.3**, the program has a user-friendly interface and a visual programmed code. The user interface allows for the input of several parameters that are easily incorporated into the program code. The environment offered by the visual programming language is user-friendly and permits the rapid identification of sources of error.



**Figure 4.3 Example screenshot of a typical polyanhydride synthesis program.** LabVIEW® permits the visual programming of robotic setups through a visual diagram flow (A). Each program can be standardized for a process asking for specific information per run to the user using a user-friendly interface in which images, buttons, etc. can be added (B).

#### **4.4.2 Synthesis of polymer and fabrication of nanoparticles**

A 50:50 CPTEG:CPH random copolymer was synthesized as described previously [5]. The molecular weight ( $M_w$ ) of the copolymer was 8,000 g/mol and  $^1\text{H}$  NMR spectra of the copolymer were consistent with previously published data [5, 9, 31]. The nanoparticles were fabricated using the robotic set up described in **Figure 4.1**. It was shown that when filtration was used as the separation method to remove pentane in the nanoparticle precipitation, less particle aggregation (average diameter =  $163 \pm 24$  nm) was observed. The yield of nanoparticles by filtration was ~50% for small-size batches (10-25 mg), while for larger batches the yield improved to ~85%. The yield by centrifugation was consistent (>90%) for both small and large batch sizes. Filtration was employed for large batches for the fabrication of nanoparticles used for functionalization since the functionalization process did not result in aggregated nanoparticles.

#### **4.4.3 Effect on nanoparticle morphology**

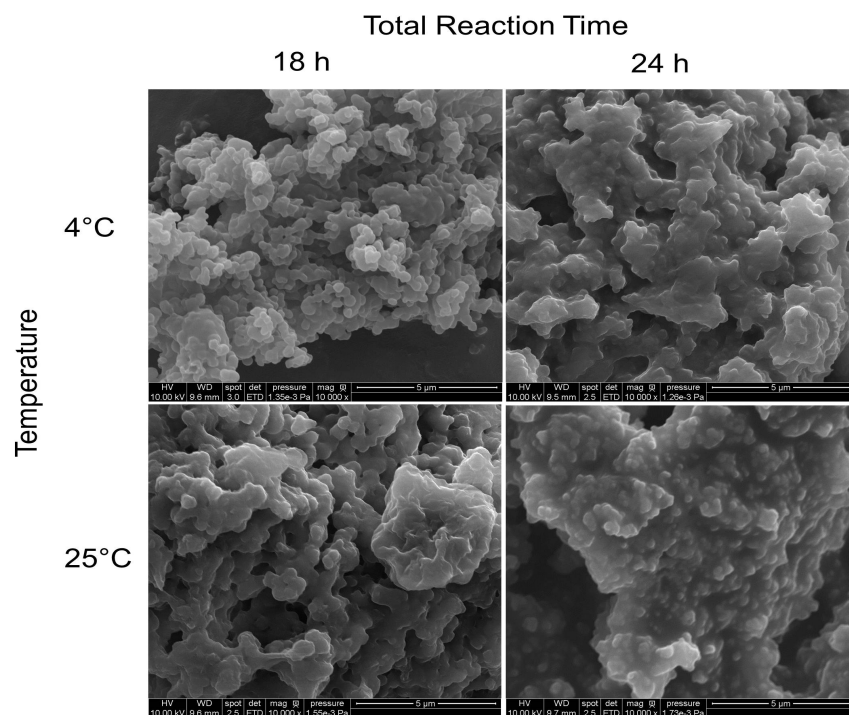
The effect of reaction temperature and time on nanoparticle morphology was profound, which is consistent with the fact that biodegradable polyanhydrides are hydrolytically labile. A qualitative assessment was performed by comparing representative SEM images of particles synthesized under different reaction conditions. It was observed that cold temperatures and short reaction times improved the morphology of the nanoparticles, preventing aggregates. In addition, the copolymer is greatly affected by the long incubation times in aqueous solution, leading to an investigation of the reduction of total reaction time. Aggregation of particles was observed at early stages of the reaction when the reaction was carried out at room temperature. The cold temperature helped delay the onset of aggregation to ~9 h of



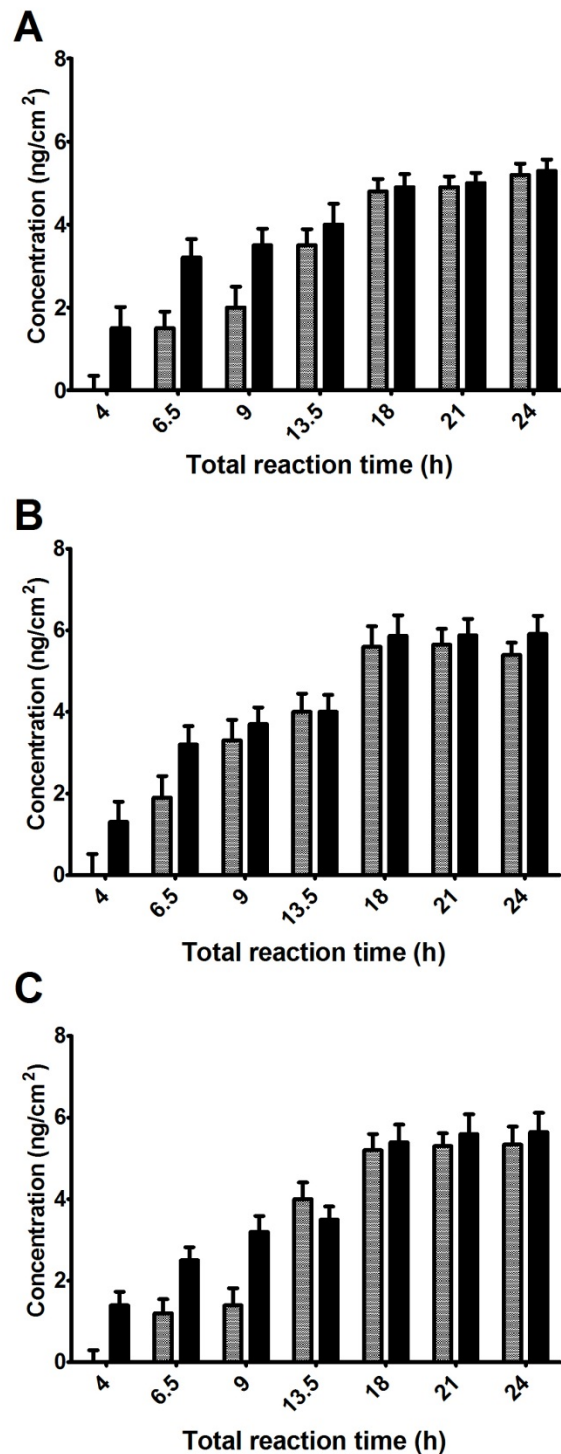
total reaction time. **Figure 4.4** shows the morphology of four experimental groups at longer total reaction times (i.e., 18 h and 24 h) at 4°C and 25°C. These photomicrographs show the detrimental effect of longer reaction times on the nanoparticle morphology and size. These results confirm that thermal properties of the materials such as the glass temperature ( $T_g$ ) are important in their processability and morphology. It has been reported that the  $T_g$  of 50:50 CPTEG:CPH is 8°C [5]. The biodegradability and phase behavior of the copolymer is another important factor to consider. Specifically, 50:50 CPTEG:CPH is an amorphous copolymer, which would lead to faster erosion rates compared to, for example, poly(CPH), which would degrade more slowly and is semi-crystalline [5].

#### 4.4.4 Effect on saccharide concentration

In the process of optimization of the amine carboxylic acid coupling reaction, the concentration of saccharide on the surface of nanoparticles is very important. The effect on saccharide concentration was studied by varying temperature and reaction time as shown in **Figure 4.5**. At cold temperatures and shorter total reaction times, the surface concentration of saccharides was lower in comparison to those of reactions carried out at room temperature. The differences in the concentration of the saccharides were less significant when comparing reactions carried out at 4°C and 25°C at longer total reaction times. The concentration of saccharides at longer reaction times did not vary greatly, when compared to the variability observed from 4 to 18 h of total reaction time at both temperatures.



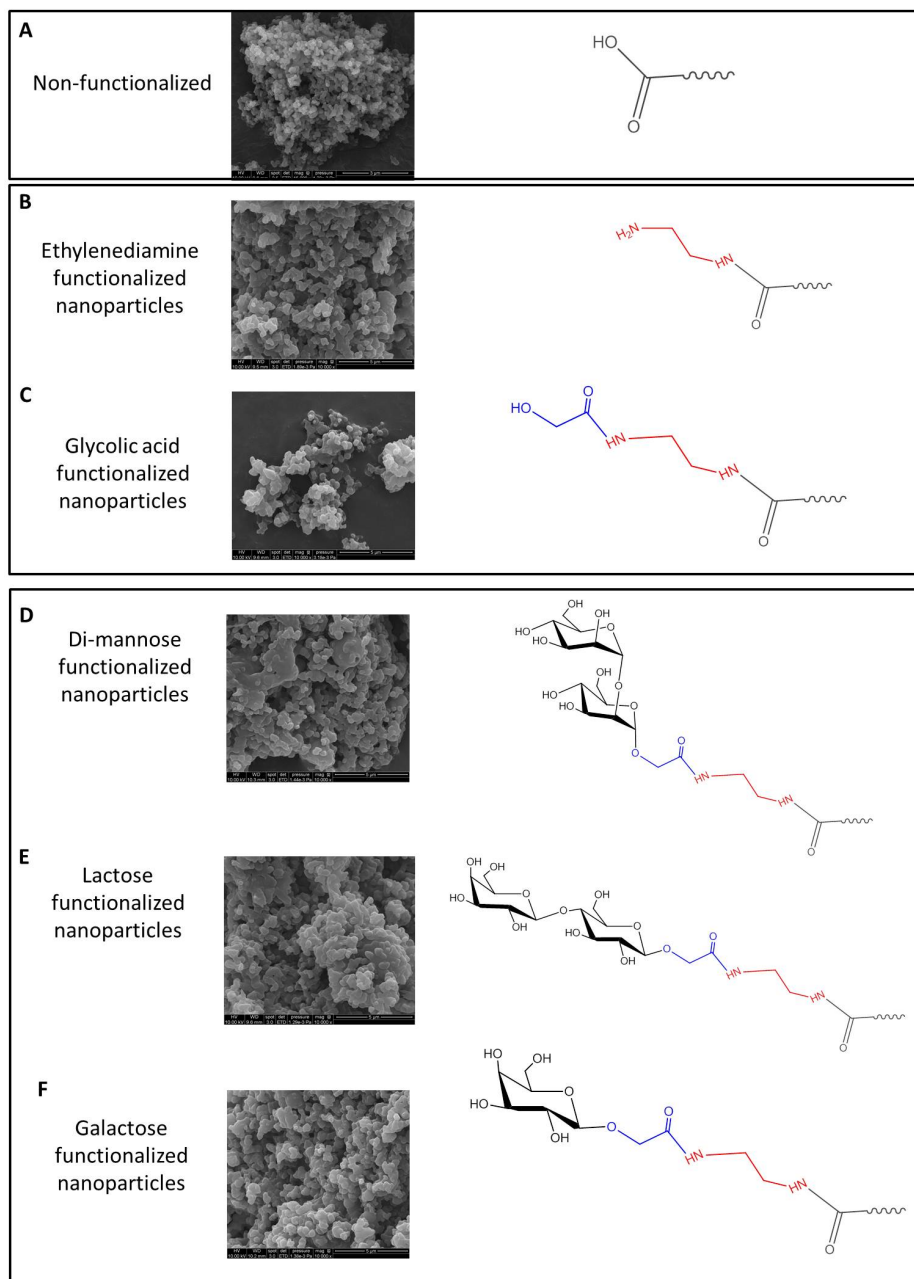
**Figure 4.4 Morphology of nanoparticles at different reaction conditions.** SEM images show the effect of temperature (4°C and 25°C) and total reaction time (18 h and 24 h) on the morphology of glycolic acid functionalized 50:50 CPTEG:CPH nanoparticles. Scale bar 5 µm.



**Figure 4.5 Effect of reaction conditions on saccharide concentration.** (A) Di-mannose, (B) Lactose and (C) Galactose. In these experiments, 50:50 CPTEG:CPH nanoparticles were used at different reaction times and temperatures: 4°C (■) and 25°C (■). The average and standard error of three independent functionalization experiments is shown.

#### 4.4.5 Optimized conditions

In summary, optimal conditions for functionalizing 50:50 CPTEG:CPH nanoparticles were found to be 18 h of total reaction time at 4°C. Under these conditions, reasonable and reproducible surface concentrations and spherical particle morphologies were observed. **Figure 4.6** shows representative SEM images of each functionalization; in all cases, aggregation was reduced by sonication. The results of the nanoparticle characterization are summarized in **Table 4.2**. The non-functionalized nanoparticles showed a negative  $\zeta$ -potential, which is characteristic of carboxylic acid motifs present on the surface of nanoparticles. After ethylenediamine was covalently linked to the nanoparticles (by the first reaction), the  $\zeta$ -potential changes to a positive value; this is expected because of the amine groups present on the surface of the particle. Glycolic acid functionalization or linker control showed a less positive surface charge. All the saccharide-functionalized nanoparticles also showed positive surface charge, indicating partial coverage of the particle surface by the saccharides. This observation is consistent with the surface coverage of the particles as shown in **Figure 4.6**.



**Figure 4.6 Optimized morphology and surface functionalization of nanoparticles.** Chemical structures of functional groups as well as SEM images of 50:50 CPTEG:CPH nanoparticles: (A) Non-functionalized, (B) Ethylenediamine, (C) Glycolic acid, (D) di-mannose, (E) lactose, and (F) galactose. Scale bar = 5  $\mu\text{m}$ .

**Table 4.2 Characterization of functionalized nanoparticles**

<b>Nanoparticle group</b>	<b>Average diameter (nm)</b>	<b><math>\zeta</math>-Potential (mV)</b>	<b>Saccharide concentration (ng/cm<sup>2</sup>)</b>
<b>Non-functionalized</b>	163 $\pm$ 24	23 $\pm$ 2.5	N/A
<b>Ethylenediamine</b>	263 $\pm$ 34	31 $\pm$ 3.6	N/A
<b>Glycolic acid</b>	261 $\pm$ 44	20 $\pm$ 2.7	N/A
<b>Di-mannose</b>	271 $\pm$ 40	28 $\pm$ 1.7	4.8 $\pm$ 0.3
<b>Lactose</b>	268 $\pm$ 51	26 $\pm$ 1.3	5.8 $\pm$ 0.7
<b>Galactose</b>	263 $\pm$ 44	24 $\pm$ 2.8	5.2 $\pm$ 0.4

N/A indicates “not applicable”.

## 4.5 Conclusions

A standardized high throughput methodology for the surface functionalization of polyanhydride nanoparticles was developed in which specific parameters were varied to build discrete libraries of functionalized nanoparticles. Several programs were developed on the LabVIEW<sup>®</sup> platform for polymer synthesis, nanoparticle fabrication, and nanoparticle functionalization in order to improve the ease and reproducibility of the process by different users. The visual programming language provided a useful tool to automate pumps and actuators. The increased throughput and reduction of process time afforded by the robotic set up enabled the rapid optimization of the functionalization of polyanhydride nanoparticles with respect to reaction temperature and total reaction time. The systematic study showed that 4°C and a total reaction time of 18 h were optimal conditions that resulted in spherical nanoparticles with equivalent concentration of saccharide attached to the surface. This methodology is reproducible, reduces batch to batch variability and user error, and allows for its use in further *in vivo* and *in vitro* studies for various applications. The methodology could also be extended to

functionalize nanoparticles with proteins, nucleic acids, other types of sugars such as high-mannosylated structures, glucan, etc., to enable research on targeting other PRRs (e.g., TLRs), cancer cells, or specific tissues.

#### **4.6 Acknowledgment**

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**CHAPTER 5: TAILORING THE IMMUNE RESPONSE BY TARGETING C-  
TYPE LECTIN RECEPTORS ON ALVEOLAR MACROPHAGES USING  
“PATHOGEN-LIKE” AMPHIPHILIC POLYANHYDRIDE  
NANOPARTICLES**

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## 5.1 Abstract

C-type lectin receptors (CLRs) offer unique advantages for tailoring immune responses. Engagement of CLRs regulates antigen presenting cell (APC) activation and promotes delivery of antigens to specific intracellular compartments inside APCs for efficient processing and presentation. In these studies, we describe a novel approach to targeted antigen delivery by decorating the surface of polyanhydride nanoparticles with specific carbohydrates to provide “pathogen-like” properties. Two conserved carbohydrate structures often found on the surface of respiratory pathogens, galactose and di-mannose, were used to functionalize the surface of polyanhydride nanoparticles and target CLRs on alveolar macrophages (AM $\phi$ ), a principle respiratory tract APC. Co-culture of functionalized nanoparticles with AM $\phi$  significantly increased cell surface expression of MHC I and II, CD86, CD40 and the CLR CIRE over non-functionalized nanoparticles. Di-mannose and galactose functionalization also enhanced the expression of the macrophage mannose receptor (MMR) and the macrophage galactose lectin (MGL), respectively. This enhanced AM $\phi$  activation phenotype was found to be dependent upon nanoparticle internalization. Functionalization also promoted increased AM $\phi$  production of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . Additional studies demonstrated the requirement of the MMR for the enhanced cellular uptake and activation provided by the di-mannose functionalized nanoparticles. These data indicate that targeted engagement of MMR and other CLRs is a viable strategy for enhancing the intrinsic adjuvant properties of nanovaccine adjuvants and promoting robust pulmonary immunity.

## 5.2 Introduction

Acute respiratory infections cause 4.25 million deaths worldwide every year [1]. A critical need exists for the development of efficacious intranasal vaccines against respiratory pathogens capable of inducing robust and protective mucosal immunity. In this regard, there is growing interest in the development of vaccines that can be easily administered at the site of infection that elicit both local and systemic immune responses [2-5].

The study of alveolar macrophages (AM $\phi$ ), a type of antigen presenting cell (APC) in the respiratory tract, is central to the development of intranasal vaccines. AM $\phi$  constitute more than 80% of the total cells obtained by bronchoalveolar lavage and they constitutively migrate from the lung to the draining lymph nodes (DLN) [6-8]. Indeed, AM $\phi$  containing bacteria appear in the lung DLN prior to the onset of pathogen-induced DC migration, thereby making them integral to the establishment of protective pulmonary immune responses [6]. AM $\phi$  are equipped to detect pathogens with the aid of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) [9]. One family of PRRs found on AM $\phi$ , known as C-type lectin receptors (CLRs), recognize conserved carbohydrate structures, including mannose and galactose, found on the surface of many respiratory pathogens, such as *Yersinia pestis*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae* and influenza viruses [10-14]. CLRs also function as phagocytic receptors and include members of the mannose receptor family and DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) [15]. Depending on the specific CLR, ligand binding initiates downstream signaling cascades that promote immune cell migration to the DLN

as well as antigen processing and presentation via MHC I and/or MHC II to prime naïve T cells [16-20].

Several research groups have explored CLR targeting as a vaccine design strategy to ensure efficient delivery of cargo to intracellular compartments that facilitate antigen processing and presentation [21-29]. Many studies have demonstrated the effectiveness of using antibodies [22] or mannoproteins from pathogens [26] to target CLRs and activate APCs. However, only a limited number report the use of carbohydrate-functionalized vaccine carriers as part of an improved adjuvant for intranasal vaccines [21, 24]. Work published by Jiang et al. indicated that alveolar macrophages could recognize mannosylated chitosan microparticles when delivered intranasally [24]. Unfortunately, mechanistic studies demonstrating the engagement of the mannose receptor on AM $\square$  by these particles were not performed.

Here, we describe functionalization of polyanhydride nanoparticles with two conserved carbohydrate structures commonly found on the surface of respiratory pathogens, di-mannose and galactose. The addition of these carbohydrates significantly enhanced the intrinsic adjuvant activity of our polyanhydride nanovaccine platform by further upregulating AM $\square$  surface expression of MHC I and II, CLRs, and T cell co-stimulatory molecules as well as secretion of pro-inflammatory cytokines. Moreover, we demonstrate that di-mannose functionalized polyanhydride nanoparticles specifically engaged the macrophage mannose receptor in order to enhance nanoparticle uptake and activate AM $\square$ .

## 5.3 Materials and Methods

### 5.3.1 Materials

The chemicals needed for monomer synthesis, polymerization and nanoparticle fabrication included 1,6-dibromohexane, triethylene glycol, 4-p-hydroxybenzoic acid, and 1-methyl-2-pyrrolidinone; these were purchased from Sigma–Aldrich (St. Louis, MO); 4-p-fluorobenzonitrile was obtained from Apollo Scientific (Cheshire, UK); toluene, sulfuric acid, acetonitrile, dimethyl formamide, acetic anhydride, methylene chloride, pentane, and potassium carbonate were obtained from Fisher Scientific (Fairlawn, NJ); p-carboxy benzoic acid (99+%), and 1-methyl-2- pyrrolidinone, anhydrous (99+%) were purchased from Aldrich (Milwaukee, WI). For  $^1\text{H}$  NMR characterization, deuterated chemicals, including chloroform and dimethyl sulfoxide, were purchased from Cambridge Isotope Laboratories (Andover, MA).

### 5.3.2 Monomer and polymer synthesis

The 1,6-bis(p-carboxyphenoxy)hexane (CPH) and 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) monomers were synthesized as previously described [30, 31]. Random copolymers with a 50:50 ratio of CPH and CPTEG were synthesized by melt polycondensation using an automated robotic deposition apparatus operated with LabVIEW® (National Instruments) as previously described [31-35]. The chemical structure of the polymers was characterized via  $^1\text{H}$  NMR with a Varian VXR 300 MHz spectrometer (Varian Inc., Palo Alto, CA). Deuterated chloroform was used to dissolve the polymer and spectra were calibrated with respect to the chloroform peak ( $\delta=7.26$  ppm). The polymer molecular mass was determined using gel permeation chromatography (GPC). Samples were dissolved in HPLC-grade chloroform and

separated on a Waters GPC chromatograph (Milford, MA) containing PL gel columns (Polymer Laboratories, Amherst, MA) comparing elution times to monodisperse polystyrene standards (Fluka, Milwaukee, WI).

### **5.3.3 High throughput synthesis of carbohydrates**

A robotic set up was used for the iterative synthesis of linear  $\alpha$ -1,2-linked di-mannose with a fluororous allyl group using fluororous solid phase extraction (FSPE) serving as a model to obtain the di-mannoside [21, 36-38]. Carboxymethyl – di-mannose synthesis was performed by ozonolysis of the alkene [39] followed by further oxidation with Jones reagent [37]. Global deprotection under Birch reduction conditions [40] produced the fully deprotected  $\alpha$ -1,2-linked di-mannose. In addition,  $\beta$ -1-O-allylated galactose was prepared from  $\beta$ -penta-O-acetylated galactose using allyl alcohol and  $\text{BF}_3 \cdot \text{OEt}_2$ . Eight equivalents of  $\text{NaIO}_4$  utilized under ruthenium-catalyzed Sharpless conditions [41, 42] produced the desired acid in 91% yield. Subsequent deacetylation under mild conditions using  $\text{K}_2\text{CO}_3$  provided the desired fully deprotected galactoside.

### **5.3.4 High throughput synthesis and characterization of functionalized nanoparticles**

The fabrication of functionalized 50:50 CPTEG:CPH nanoparticles was performed via an anti-solvent nanoencapsulation method using an automated robotic deposition apparatus operated by LabView<sup>®</sup> [32, 33, 43]. Galactose and di-mannose residues were conjugated to the surface of nanoparticles by a modified and optimized two-step amine carboxylic acid coupling reaction [21]. Briefly, the first reaction was performed at 4°C by incubating the nanoparticle suspension (100 mg/mL) with 12 equivalents (eq.) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride



(EDC), 12 eq. of *N*-hydroxysuccinimide (NHS), and 10 eq. of ethylenediamine in nanopure water for 9 h with constant agitation using a rotor shaker (Scientific Industries, Bohemia, NY). Nanoparticles were washed twice by centrifugation (10,000 rpm for 5 min) with the addition of nanopure water and sonication (at 40 Hz for 30 s). The second reaction was performed at 4°C in a nanoparticle suspension (50 mg/mL) in nanopure water with 12 eq. of EDC, 12 eq. of NHS and 10 eq. of the corresponding saccharide (i.e., galactose or di-mannose) or glycolic acid (linker between saccharide and nanoparticles; a control treatment for AM $\square$  experiments) for 9 h with constant agitation. Nanoparticles were washed once and dried under vacuum for 6 h. The automated set up was used to accurately dispense solutions of EDC, ethylenediamine, and NHS for the first reaction, and solutions of EDC, NHS, and saccharides in the second reaction to increase the throughput of the process. Particle morphology was characterized by scanning electron microscopy (SEM, FEI Quanta 250, Kyoto, Japan), and hydrodynamic size and  $\zeta$ -potential were determined by dynamic light scattering (DLS, Zetasizer Nano, Malvern Instruments Ltd., Worcester, UK). The saccharide concentration conjugated to the nanoparticles was measured using a high throughput phenol sulfuric acid assay as previously described [21, 44].

### 5.3.5 Mice

Wild type (WT) C57BL/6 (B6) mice were purchased from Harlan Laboratories (Indianapolis, IN) and macrophage mannose receptor deficient (MMR<sup>-/-</sup>) B6 mice were a generous gift from Dr. Mary Ann McDowell of the University of Notre Dame. Mice were housed in specific pathogen-free conditions where all bedding, caging, and feed were sterilized prior to use. All animal procedures were conducted with the approval of the

Iowa State University Institutional Animal Care and Use Committee.

### 5.3.6 Cell harvesting and culture

Murine alveolar macrophages (AM $\square$ ) from WT and MMR<sup>-/-</sup> B6 mice were harvested by bronchoalveolar lavage as previously described [6, 7, 45]. Briefly, mice were euthanized and a sterile catheter inserted into the trachea of each mouse. Using a 1 mL syringe fitted with the catheter, 0.75 mL of room temperature, sterile PBS was gently infused into the lungs and then aspirated back into syringe. This process was repeated six times while externally massaging the chest. Following collection, lavage fluid was immediately placed on ice prior to centrifugation (250 x g, 10 min, 4°C). Cell viability was then assessed using trypan blue. Cells were cultured in Dulbecco's Modified Eagle Medium containing 4.5 mg of glucose/mL, 2 mM L-glutamine, 100 U penicillin, 100  $\mu$ g streptomycin/mL, 25 mM HEPES and 10% fetal bovine serum in six-well plates at a density of  $5 \times 10^5$  cells/well for 8 h at 37°C and 5% CO<sub>2</sub>. After 6 h of incubation, non-adherent cells were discarded and adherent cells (>90%) were incubated overnight prior to treatment. Non-functionalized or functionalized nanoparticles were incubated with AM $\square$  at a concentration of 0.125 mg/mL. Non-stimulated AM $\square$  and AM $\square$  stimulated with *Escherichia coli* lipopolysaccharide (LPS, 200 ng/mL Sigma Aldrich, St. Louis, MO) were used as negative and positive controls respectively. After 48 h, supernatants were collected for quantification of cytokines and nitrites. Cells were stained for flow cytometric analysis.

### 5.3.7 Cell surface marker evaluation

Flow cytometric evaluation of cell surface markers was performed by modifying a previously described protocol [46]. Briefly, cells were washed in 2 mL of fluorescence-

activated cell sorting buffer (FACS, 0.1% sodium azide and 0.1% bovine serum albumin in phosphate buffer saline). Fcγ receptors were blocked with 10% purified rat anti-mouse CD16/CD32 antibody (BD Bioscience, San Diego, CA) in 1 mg/mL rat IgG for 30 min at 4°C to prevent non-specific binding. AM $\square$  were incubated with appropriate antibodies or isotype controls for 15 min on ice. Antibodies used for assessment of activation included phycoerythrin (PE)-Cy7 conjugated anti-mouse F4/80 (clone BM8), fluorescein isothiocyanate (FITC)-conjugated anti-mouse/rat MHC II (I-A/I-E, clone M5/114.15.2), allophycocyanin (APC) anti-mouse CD40 (clone 1C10) and PE conjugated anti-mouse MHC I (H-2Kb, clone AF6-88.5.5.3). These antibodies and their respective isotype controls were purchased from eBioscience (San Diego, CA). APC-Cy7 anti-mouse CD86 (clone GL-1) was purchased from Biolegend (San Diego, CA). Antibodies used to evaluate CLR expression included F4/80, biotin conjugated anti-mouse CD209 (clone 5H10) and PE-Texas red conjugated streptavidin purchased from BD Biosciences, FITC anti-mouse CD206 (MMR, clone MR5D3) purchased from Biolegend and PE-CD301a/b (MGL1/2, affinity purified PAb catalog # FAB4297P) purchased from R&D systems (Minneapolis, MN). Samples were acquired using a FACS Aria III flow cytometer (BD Biosciences) and data was analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

### **5.3.8 Nanoparticle internalization**

Nanoparticles were loaded with cadmium selenide quantum dots (QDs; emission at 630 nm) prior to functionalization. The QDs were kindly provided by Dr. Aaron Clapp, Iowa State University. QD-loaded functionalized and non-functionalized nanoparticles were used to stimulate AM $\square$  for 48h. Cells were then analyzed by flow cytometry to

identify the populations of nanoparticle-positive and nanoparticle-negative cells as described previously [47]. A QD control (background) was used to account for “false positives” because of QDs released due to particle degradation. QD-loaded functionalized and non-functionalized nanoparticles were incubated in cell culture media for 48 h. After centrifugation (250 x g, 10 min, 4°C), supernatants were added to AM $\square$ . After 48 h, the fluorescence registered for these control groups was considered as background. Cells were labeled with the above-described F4/80, MHC II, CD40, CD86 and CD301a/b antibodies as wells as with Pacific blue anti-mouse MHC I (H-2Kb, clone AF6-88.5) purchased from Biolegend, biotin anti-mouse CD209 and PerCP-Cy5.5 conjugated streptavidin purchased from eBioscience.

### **5.3.9 Cytokine and reactive nitrogen species assays**

After stimulation for 48 h with non-functionalized or functionalized nanoparticles, cell-free supernatants were assayed for IL-1 $\beta$ , IL-10, TNF- $\alpha$ , IL-6, and IL-12p40 using a multiplex cytokine assay in conjunction with a Bioplex 200 System (Bio-Rad, Hercules, CA) as described elsewhere [33]. Griess Reagent (Invitrogen, Carlsbad, CA) was used to determine nitrite concentration as an indirect method to measure reactive nitrogen species.

### **5.2.11 Statistical analysis**

The statistical software JMP<sup>®</sup>7 was used to analyze all data. Tukey’s HSD was used to determine significant differences among treatments. A student’s T test was employed to determine the significant differences between AM $\square$  harvested from WT versus MMR<sup>-/-</sup> mice and between nanoparticle positive and negative AM $\square$ . All p values < 0.05 were considered significant.

## 5.4 Results

### 5.4.1 Synthesis and characterization of functionalized polyanhydride nanoparticles.

Amphiphilic 50:50 CPTEG:CPH copolymer was synthesized as described previously [31]. The molecular weight ( $M_w$ ) of the copolymer was 8,000 g/mol and  $^1\text{H}$  NMR spectra of the copolymer were consistent with previously published data [31, 33, 35]. Particle morphology was evaluated by SEM (data not shown). The average diameter of the non-functionalized nanoparticles was  $163 \pm 24$  nm with a  $\zeta$ -potential of  $-23 \pm 2.5$  mV (consistent with the presence of carboxylic acids on the surface). The glycolic acid (linker only) functionalized nanoparticles had an average diameter of  $261 \pm 44$  nm and a  $\zeta$ -potential of  $20 \pm 2.7$  mV, while di-mannose functionalized and galactose functionalized nanoparticles had average diameters of  $271 \pm 40$  nm and  $263 \pm 44$  nm, respectively, with similar  $\zeta$ -potentials of  $26 \pm 1.9$  mV. The positive charges of the functionalized particles were consistent with previously published data [21] and are attributed to the presence of free ethylenediamine groups on the surface of the particles. Measuring the  $\zeta$ -potential of ethylenediamine-functionalized nanoparticles (determined to be  $31 \pm 3.6$  mV) further corroborated this result. These data indicate that partial capping of the ethylenediamine groups occurs when the saccharide is conjugated in the second reaction of the process. The di-mannose and galactose concentrations attached to the nanoparticles were normalized to the total mass of nanoparticles. The di-mannose concentration was  $13.2 \pm 3.5$   $\mu\text{g}/\text{mg}$ , consistent with previous work [21]. The galactose concentration conjugated to the nanoparticles was  $15.2 \pm 4.7$   $\mu\text{g}/\text{mg}$ .

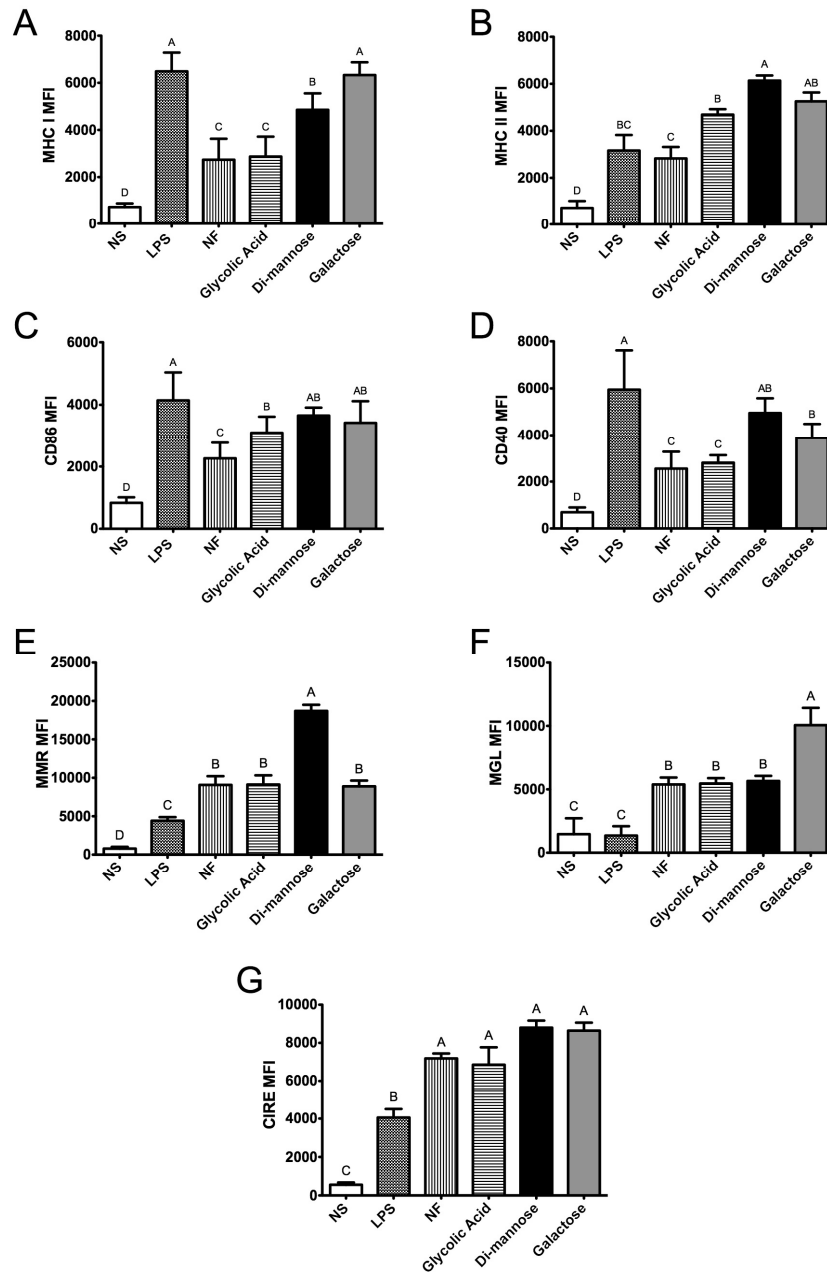
#### **5.4.2 Functionalization of polyanhydride nanoparticles enhanced AM $\phi$ expression of MHC, T cell co-stimulatory molecules and CLRs.**

Previous works from our laboratories have highlighted the intrinsic adjuvant activity of polyanhydride particles, as evidenced by their ability to activate DCs [46, 47]. In the current study, we observe, for the first time, enhanced expression of surface markers associated with antigen processing and presentation (MHC I and II) and T-cell co-stimulation (CD86 and CD40) in primary AM $\phi$  cultured with non-functionalized polyanhydride nanoparticles as compared to non-stimulated AM $\phi$  (**Figure 5.1A-D**). Culture of AM $\phi$  with non-functionalized polyanhydride nanoparticles also enhanced surface expression of CLRs (MMR, MGL and CIRE) in comparison to non-stimulated AM $\phi$  and AM $\phi$  stimulated with LPS (**Figure 5.1E-G**).

Functionalization of polyanhydride nanoparticles with either di-mannose or galactose provided a significant upregulation in the expression of MHC I and CD40 on the surface of AM $\phi$  in comparison to treatment with either non-functionalized particles or particles functionalized with only glycolic acid, the linker used to attach the carbohydrates to the nanoparticles (**Figures 5.1A and 5.1D**). AM $\phi$  MHC II and CD86 expression were enhanced over non-functionalized particles regardless of the nanoparticle functionalization (**Figures 5.1B and 5.1C**), with the greatest enhancement in MHC II observed on AM $\phi$  cultured with di-mannose functionalized particles (**Figure 5.1B**). As compared to non-functionalized nanoparticles, expression of the MMR on AM $\phi$  was only significantly enhanced upon culture with di-mannose functionalized nanoparticles (**Figure 5.1E**) while only culture with galactose functionalized nanoparticles significantly increased AM $\phi$  MGL expression (**Figure 1F**). No significant

enhancement in CIRE expression was observed when AM $\phi$  were cultured with functionalized versus non-functionalized nanoparticles (**Figure 1G**).

Together, these observations indicate that carbohydrate functionalization of polyanhydride nanoparticles enhanced AM $\phi$  activation as compared to non-functionalized particles. In some instances (for MHC II and CD86 expression), functionalization with only the glycolic acid linker increased AM $\phi$  activation, indicating that nanoparticle surface charge contributes to AM $\phi$  activation. Finally, AM $\phi$  culture with di-mannose or galactose functionalized particles provided enhanced cellular activation profiles that were comparable or even superior to those observed in AM $\phi$  stimulated with LPS.



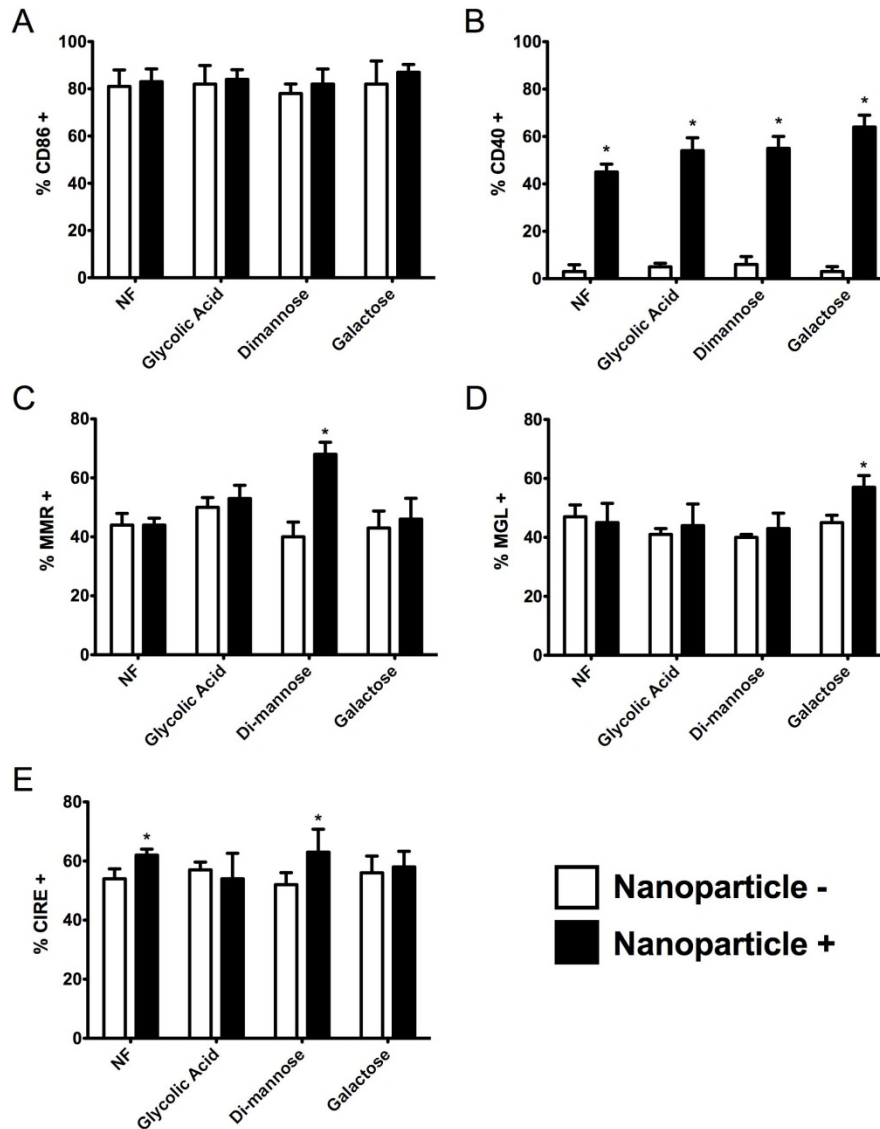
**Figure 5.1 Functionalization of polyanhydride nanoparticles enhanced AM $\phi$  expression of MHC, T cell co-stimulatory molecules and CLRs.** After stimulation with non-functionalized (NF) or functionalized nanoparticles for 48 hrs, AM $\phi$  were harvested and analyzed by flow cytometry for surface expression of (A) MHC I, (B) MHC II, (C) CD86, (D) CD40, (E) MMR, (F) MGL, or (G) CIRE. LPS stimulated and non-stimulated cells (NS) were used as positive and negative controls, respectively. Data are expressed as the mean  $\pm$  the SEM of three independent experiments performed in triplicate. Treatments with different letters are significantly different from one another at  $p < 0.05$ . MFI = mean fluorescence intensity.



### **5.4.3 Internalization of functionalized polyanhydride nanoparticles was required for upregulation of CD40 expression on AM $\phi$**

Pathogen phagocytosis is an important step in activating macrophages [48, 49]. We therefore evaluated the relationship between nanoparticle internalization and AM $\phi$  activation using quantum dot-loaded nanoparticles and flow cytometry [47]. Internalization of, and not just association with, nanoparticles by AM $\phi$  was confirmed by confocal microscopy (data not shown). Two populations of cells were identified—cells that internalized QD-loaded nanoparticles (nanoparticle-positive) and cells that did not internalize particles (nanoparticle-negative). Internalization of nanoparticles, regardless of functionalization status, was found to be required for the enhanced expression of CD40 on AM $\phi$  (**Figure 5.2B**), but not for the expression of CD86, MHC I, and MHC II (**Figure 5.2A and data not shown**).

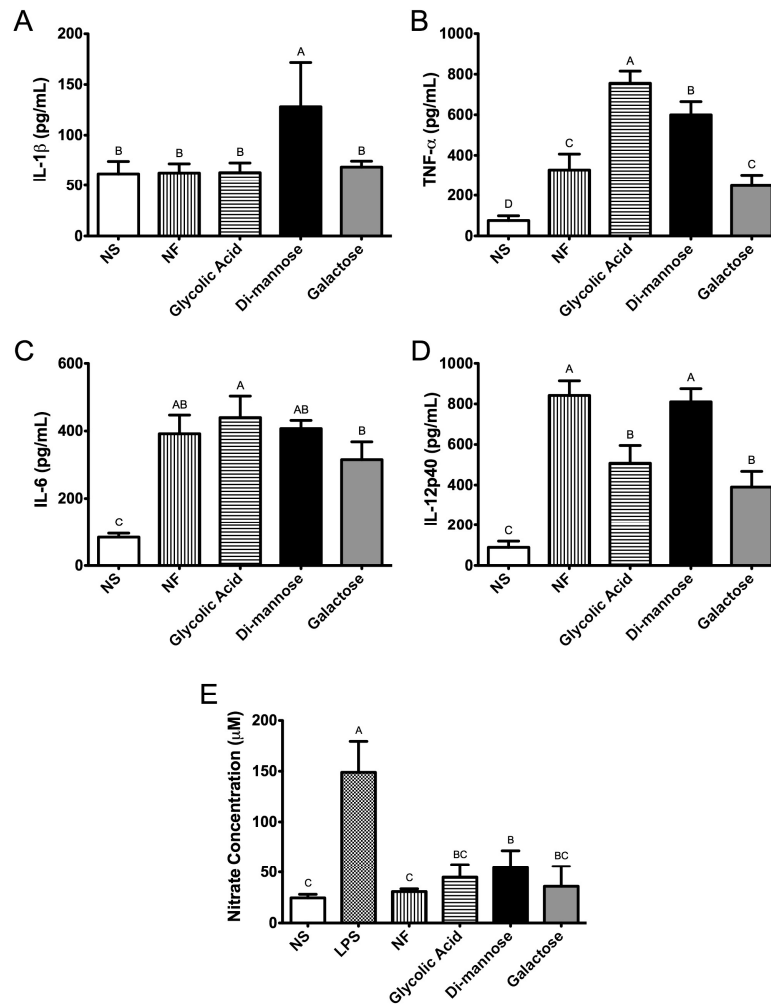
Interestingly, the nanoparticle-positive cells that internalized di-mannose but not galactose-functionalized nanoparticles expressed significantly greater levels of the MMR on their surface as compared to nanoparticle-negative cells (**Figure 5.2C**). A similar relationship was observed for galactose but not di-mannose functionalized particles and MGL expression (**Figure 5.2D**). These data support an association between internalization of nanoparticles functionalized with a certain carbohydrate and the enhanced expression of the receptor specific for that carbohydrate.



**Figure 5.2 Internalization of functionalized polyanhydride nanoparticles was required for upregulation of CD40 expression on AM.** Percent of AM that internalized (nanoparticle-positive; ■) or did not internalize (nanoparticle-negative; □) nanoparticles after 48 hrs that were also positive for (A) CD86, (B) CD40, (C) MMR, (D) MGL, or (E) CIRE. Data are expressed as the mean  $\pm$  the SEM of three independent experiments performed in triplicate. \* represents a statistically significant difference between nanoparticle-positive and nanoparticle-negative populations within a treatment at  $p < 0.05$ .

#### **5.4.4 Carbohydrate functionalization of nanoparticles differentially influenced proinflammatory cytokine secretion**

Previous reports from our laboratory have described the ability of non-functionalized polyanhydride nanoparticles to enhance secretion of pro-inflammatory cytokines from APCs [32, 46]. In this present work, we sought to extend our findings by asking if functionalization with specific carbohydrates provides additional stimulatory capacity to the polyanhydride nanoparticles. Indeed, di-mannose functionalized nanoparticles significantly enhanced the production of IL-1 $\beta$  from AM $\phi$  as compared to all nanoparticle treatments (**Figure 5.3A**). Functionalization with either di-mannose or the glycolic acid linker but not galactose enhanced AM $\phi$  secretion of TNF- $\alpha$  (**Figure 3B**). Di-mannose modification of the nanoparticles provided no additional benefit in terms of enhancing IL-6 and IL-12p40 production (**Figures 5.3C and 5.3D**). Of note, functionalization with either the glycolic acid linker only or galactose diminished the enhanced IL-12p40 secretion observed when AM $\phi$  were co-cultured with non-functionalized nanoparticles (**Figure 5.3D**). No IL-10 was detected in the supernatants of any AM $\phi$  and nanoparticle co-cultures (**data not shown**).



**Figure 5.3 Carbohydrate functionalization of nanoparticles differentially influenced pro-inflammatory cytokine secretion.** After stimulation with non-functionalized (NF) or functionalized nanoparticles for 48 hrs, culture supernatants were harvested and assayed for (A) IL-1 $\beta$ , (B) TNF- $\alpha$ , (C) IL-6, and (D) IL-12p40. LPS stimulated and non-stimulated cells (NS) were used as positive and negative controls, respectively. Mean cytokine production for AM $\square$  stimulated with LPS: IL-1 $\beta$  = 1,021.7  $\pm$  66.5 pg/mL, IL-6 = 7,804.3  $\pm$  101.2 pg/mL, TNF- $\alpha$  = 5,711.5  $\pm$  181.7 pg/mL, and IL-12p40 = 3,637.8  $\pm$  111.6 pg/mL. (F) Nitrate concentration was measured in culture supernatants via a Griess assay as an indirect method to quantify production of reactive nitrogen species. Data are expressed as the mean  $\pm$  the SEM of three independent experiments performed in triplicate. Treatments with different letters are significantly different from one another at  $p < 0.05$ .

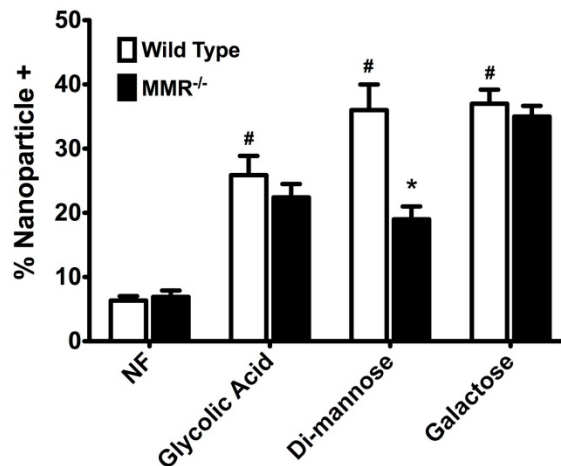
#### **5.4.5 Di-mannose functionalized nanoparticles enhanced internalization and AM $\phi$ activation by engaging the macrophage mannose receptor.**

In **Figure 5.2**, we observed that nanoparticle internalization was required for increased CD40 and CLR expression. We next performed additional experiments to determine if the enhanced activation of AM $\phi$  by functionalized nanoparticles seen in **Figures 5.1 and 5.3** was associated with enhanced nanoparticle internalization. Using quantum dot-loaded nanoparticles and flow cytometry, we found that any functionalization of the nanoparticle surface enhanced uptake by AM $\phi$  as compared to non-functionalized particles (**Figure 5.4, white bars**).

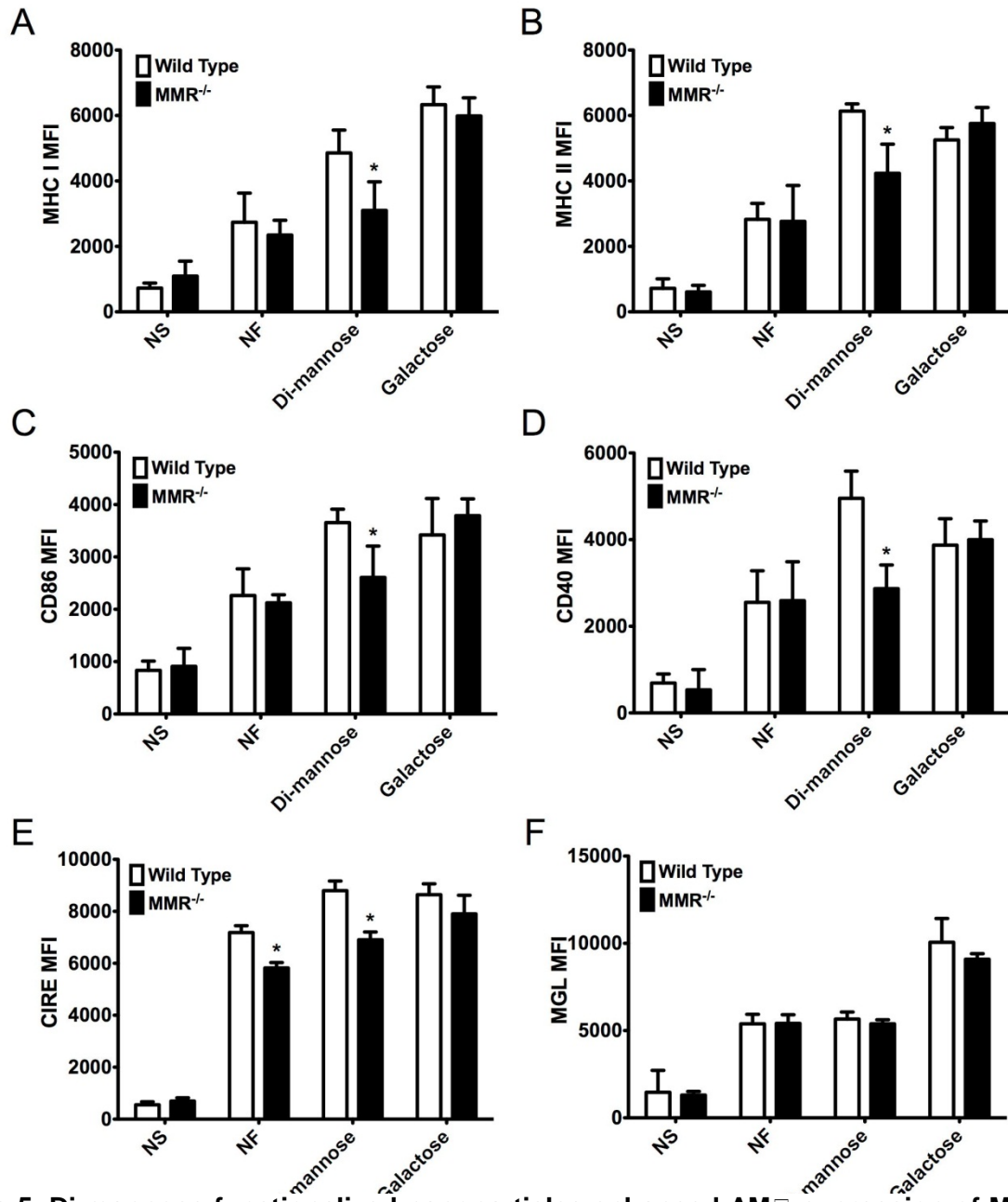
To test the specificity of the di-mannose modification for binding to the MMR, we isolated AM $\phi$  from MMR-deficient (MMR<sup>-/-</sup>) mice and co-cultured them with functionalized nanoparticles. As compared to wild type (WT) AM $\phi$ , a significant decrease in the number of internalized nanoparticles was only observed when di-mannose functionalized nanoparticles were co-cultured with MMR<sup>-/-</sup> AM $\phi$  (**Figure 5.4, white versus black bars**). These data indicate that di-mannose functionalized nanoparticles specifically interact with the MMR, while nanoparticles functionalized with galactose or only the glycolic acid linker are internalized via other, non-MMR-dependent pathways. Similar results were obtained using bone marrow-derived macrophages (BMM $\phi$ ; **Supplemental Figure 5.1**).

Consistent with the internalization data, only the MMR<sup>-/-</sup> AM $\phi$  co-cultured with di-mannose functionalized nanoparticles failed to enhance surface expression of MHC I, MHC II, CD86 and CD40 to levels consistent with those observed for WT AM $\phi$  (**Figures 5.5A – D**). Similarly, MMR<sup>-/-</sup> AM $\phi$  only secreted significantly less IL-1 $\beta$  and IL-6 as

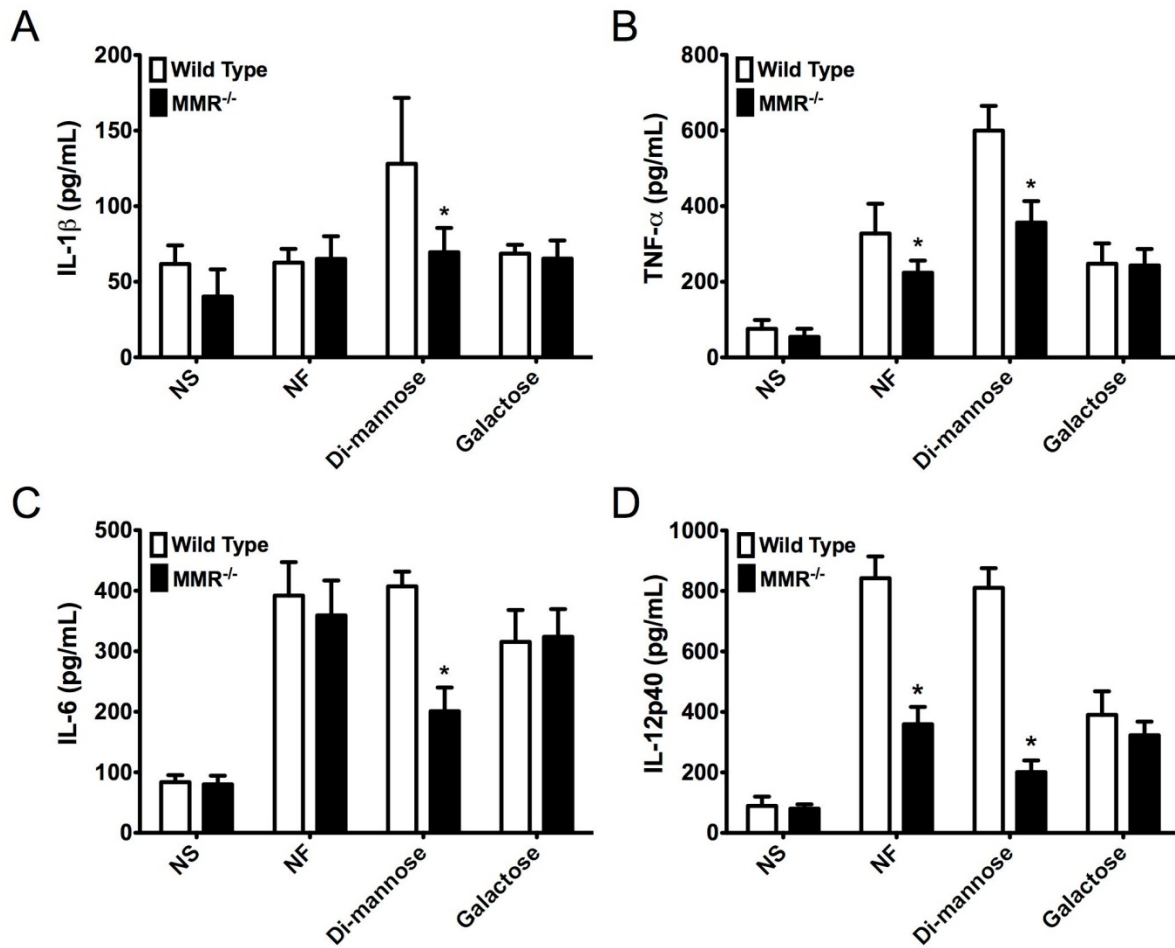
compared to WT AM $\phi$  when co-cultured with di-mannose functionalized nanoparticles (**Figures 6A and 6C**). As compared to WT AM $\phi$ , reduced levels of CIRE expression (**Figure 5E**) and TNF- $\alpha$  and IL-12p40 secretion (**Figures 5. 6B and 5.6D**) were also observed in MMR $^{-/-}$  AM $\phi$  co-cultured with di-mannose functionalized as well as with non-functionalized nanoparticles. The absence of the MMR had no negative effect on the increased surface marker expression and cytokine production observed when AM $\phi$  were co-cultured with galactose functionalized nanoparticles (**Figures 5.5 and 5.6**), indicating that galactose functionalized particles do not engage the MMR to promote AM $\phi$  activation. Similar observations were made using BMM $\phi$  (**Supplemental Figures 5.2 and 5.3**). Together, these data support the concept that di-mannose functionalized nanoparticles enhance AM $\phi$  activation by specifically engaging the MMR.



**Figure 5.4 Di-mannose functionalized nanoparticles enhanced internalization by engaging the macrophage mannose receptor on AM $\phi$ .** Percent of wild type (□) and MMR-deficient (MMR $^{-/-}$ ; ■) AM $\phi$  that internalized nanoparticles after 48 hrs. Data are expressed as the mean  $\pm$  the SEM of three independent experiments performed in triplicate. \* represents a statistically significant difference between wild type and MMR $^{-/-}$  AM $\phi$  within a treatment at  $p < 0.05$ . # represents a statistically significant difference from the non-functionalized nanoparticle treatment group for wild type AM $\phi$ .



**Figure 5. Di-mannose functionalized nanoparticles enhanced AM expression of MHC, T cell co-stimulatory molecules and CLRs by engaging the macrophage mannose receptor.** After stimulation with non-functionalized (NF) or functionalized nanoparticles for 48 hrs, wild type (□) and MMR-deficient (MMR<sup>-/-</sup>; ■) AM were harvested and analyzed by flow cytometry for surface expression of (A) MHC I, (B) MHC II, (C) CD86, (D) CD40, (E) MGL, or (F) CIRE. Data are expressed as the mean ± the SEM of three independent experiments performed in triplicate. \* represents a statistically significant difference between wild type and MMR<sup>-/-</sup> AM within a treatment at  $p < 0.05$ . MFI = mean fluorescence intensity.



**Figure 6. Di-mannose functionalized nanoparticles enhanced AM pro-inflammatory cytokine production by engaging the macrophage mannose receptor.** After stimulation with non-functionalized (NF) or functionalized nanoparticles for 48 hrs, culture supernatants from wild type (□) and MMR-deficient (MMR<sup>-/-</sup>; ■) AM were harvested and assayed for (A) IL-1β, (B) TNF-α, (C) IL-6, and (D) IL-12p40. Non-stimulated cells (NS) were used as a negative control. Data are expressed as the mean ± the SEM of three independent experiments performed in triplicate. \* represents a statistically significant difference between wild type and MMR<sup>-/-</sup> AM within a treatment at p < 0.05.



## 5.5 Discussion

In the present work, we describe a novel approach to targeted antigen delivery by decorating the surface of polyanhydride nanoparticles with specific carbohydrates to provide “pathogen-like” properties that ensure nanoparticles engage C-type lectin receptors on AM $\phi$  to induce robust immune responses in the respiratory tract. The surface of polyanhydride nanoparticles was functionalized by covalent linkage of di-mannose and galactose residues. Co-culture of functionalized nanoparticles with AM $\phi$  significantly increased cell surface expression of MHC I and II, CD86, CD40 and the C-type lectin receptor CIRE over non-functionalized nanoparticles (**Figures 5.1A-D and 1G**). Di-mannose and galactose functionalization also enhanced the expression of the MMR and MGL, respectively (**Figures 5.1E and 1F**). Carbohydrate modification also significantly increased uptake of the nanoparticles by AM $\phi$ . Moreover, the enhanced expression of CD40, CIRE, MMR and MGL on AM $\phi$  cultured with functionalized nanoparticles was found to be dependent upon nanoparticle internalization (**Figure 5.2**). In addition, functionalization promoted increased AM $\phi$  production of IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . Additional studies demonstrated the requirement of the MMR for the enhanced cellular uptake and activation provided by the di-mannose functionalized nanoparticles (**Figure 5.4-5.6**).

Di-mannose and galactose functionalized nanoparticles were engineered with an eye towards targeting specific receptors such as CIRE, MMR, and MGL. Our results show that the targeting of these specific receptors, known for their ability to internalize antigens and present them in MHC I and/or MHC II compartments [50-52], resulted in a higher percentage of cells that internalize nanoparticles (**Figure 5.4, open bars**). This

enhanced internalization may improve antigen delivery to AM $\phi$  and result in the enhancement of the expression of activation markers (i.e., MHC I, MHC II, CD86, and CD40) as shown in **Figure 5.1**. Our studies also evaluated the relationship between nanoparticle internalization and cell surface marker expression (**Figure 5.2A and 5.2B**). Simply the presence of the nanoparticles in the co-culture was sufficient for enhancement of certain activation markers (i.e. CD86, MHC I, and MHC II), while internalization was required for the expression of other activation makers, including CD40.

Nanoparticle internalization also played a critical role in the enhanced expression of CLRs (**Figures 5.2C-E**). MMR expression was enhanced on AM $\phi$  following co-culture with mannose-functionalized nanoparticles (**Figure 5.1E**), and this enhanced expression required internalization of the nanoparticles (**Figure 5.2C**). Similar results were observed for MGL (**Figures 5.1F and 5.2D**). The expression of the CIRE receptor was enhanced by most of the nanoparticle groups, but only cells that internalized non-functionalized and di-mannose functionalized nanoparticles showed enhanced expression of this marker (**Figures 5.1G and 5.2E**). Several reports have shown that CLRs undergo internalization and recycling between the plasma membrane and the endosomal compartments [53-55]. Our results suggest the presence of a feedback loop that increases the expression of these markers after ligand recognition.

The production of pro-inflammatory cytokines is essential for the activation of macrophages as initiators of an adaptive immune response [56]. Di-mannose functionalization enhanced the production of IL-12p40, IL-1 $\beta$  and TNF- $\alpha$  (**Figures 5.3A-D**). These results are in agreement with studies demonstrating that the targeting of

MMR resulted in the production of pro-inflammatory cytokines through the activation of NF- $\kappa$ B [57]. Although the production of pro-inflammatory cytokines was enhanced by the di-mannose functionalization, the levels secreted were low enough to allay concerns about a chronic inflammation event in the respiratory tract. The galactose functionalized nanoparticles resulted in an enhanced production of cytokines in comparison with the negative control, but at lower levels than the non-functionalized nanoparticles, particularly for IL-12p40. The release of pro-inflammatory cytokines when the cells were treated with galactose-functionalized particles showed was similar to that of the non-functionalized nanoparticles. Previous studies suggest that galactose motifs enhanced the production of cytokines such as IL-12p40 and TNF- $\alpha$ , but not to the levels obtained when mannose motifs are used [57, 58].

The constant production of reactive nitrogen species is related to chronic inflammation and cancers of several organs, including lungs [59]. Such a response may be needed to combat an infection; however it would not be desirable in an intranasal vaccine. As shown in **Figure 5.3E**, all the nanoparticle groups have similar levels of reactive nitrogen species production in comparison with the non-stimulated group (negative control). Together, these data demonstrate that di-mannose functionalization significantly enhanced the expression of cell surface makers and the production of relevant cytokines. These events could promote a suitable phenotype of AM $\phi$  that could trigger an enhanced T cell activation, which is an important outcome for intranasal vaccines.

To further assess the engagement of MMR in the specific recognition of di-mannose functionalized nanoparticles, studies were performed on AM $\phi$  harvested from

MMR KO mice. Statistically significant differences were observed in the internalization of di-mannose functionalized nanoparticles between AM $\phi$  harvested from wild type and MMR KO mice (**Figure 5.4**), indicating that the enhanced uptake observed when polyanhydride nanoparticles are functionalized occurs via MMR-mediated endocytosis. Enhanced uptake was also seen for glycolic acid functionalized nanoparticles as compared to non-functionalized nanoparticles. This may be attributed to the hydrophilic properties conferred by the mannose and glycolic acid groups to the surface of the particles that may increase their internalization in comparison to more hydrophobic surfaces (i.e., non-functionalized nanoparticles). This observation is consistent with previously published data that shows that hydrophilic chemistries are more readily internalized by AM $\phi$  and DCs [47, 60]. As expected, the absence of the MMR did not negatively affect the enhanced uptake of galactose-functionalized nanoparticles. The enhanced AM $\phi$  activation profile observed following co-culture with di-mannose functionalized nanoparticles was also found to be dependent upon the presence of the MMR (**Figures 5.5 and 5.6**). This suggests that not only does the MMR promote internalization of the di-mannose functionalized, but also that engagement of the MMR further enhances the expression of cell surface markers and cytokine production. Taken together, these results imply a specific engagement of the receptor in the recognition of di-mannose functionalized nanoparticles and in the downstream cascade of events that promotes the cytokine production. This indicates that the di-mannose functionalized nanoparticles have “pathogen-like” capabilities with respect to their internalization and APC activation capabilities.

Together, these studies indicate that targeting CLRs is an effective strategy in the design of efficacious intranasal vaccines. In addition, enhanced expression of activation markers initiating an immune response and a positive feedback loop with enhanced cytokine production can be achieved differently depending on the specific receptor engaged in recognizing the functionalized groups. The functionalized nanoparticles provide a versatile and robust platform that can be used to tailor the immune response. Finally, the ligands can be combined, via the use of cocktails of differently functionalized polyanhydride nanoparticles, which may trigger a specific set of PRRs and generate a potent immune response, much like that induced by pathogens. This mode of activating the immune system represents a viable strategy in the rational design of efficacious intranasal vaccines.

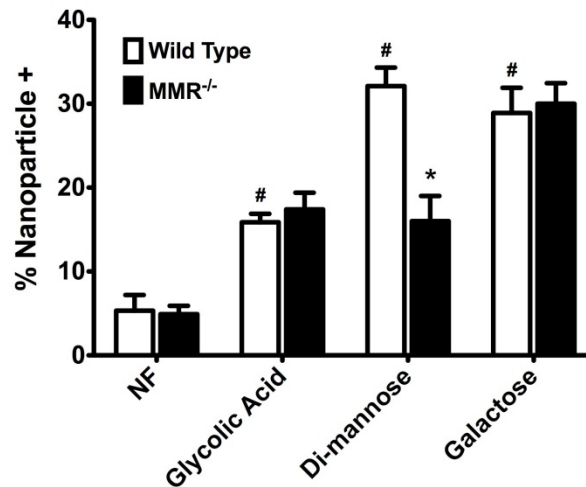
## 5.6 Conclusions

The approach outlined in this present work demonstrates that rational design of efficacious vaccine adjuvants can be achieved by targeting CLRs on APCs. Specifically, we describe the functionalization of polyanhydride nanoparticles with two conserved carbohydrate structures commonly found on the surface of respiratory pathogens, di-mannose and galactose. The addition of these carbohydrates significantly enhanced the intrinsic adjuvant activity of our polyanhydride nanovaccine platform by further upregulating AM surface expression of MHC I and II, CLRs, and T cell co-stimulatory molecules as well as secretion of pro-inflammatory cytokines. Moreover, we demonstrate that di-mannose functionalized polyanhydride nanoparticles specifically engaged the macrophage mannose receptor in order to enhance nanoparticle uptake

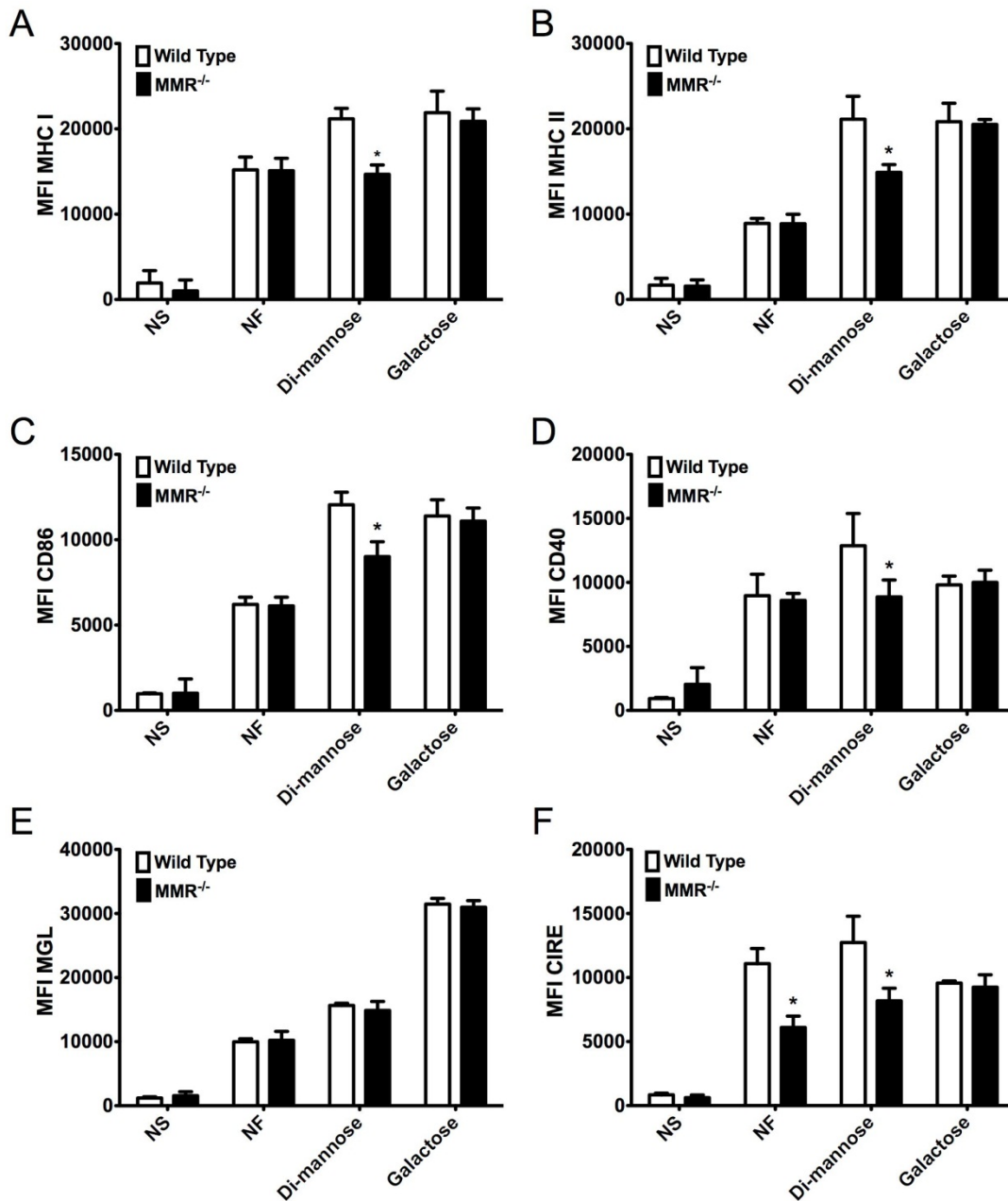
and activate AM $\square$ . These studies provide important insights into the design and rational selection of potential intranasal vaccine candidates that can be modified to improve their adjuvanticity.

## 5.7 Acknowledgments

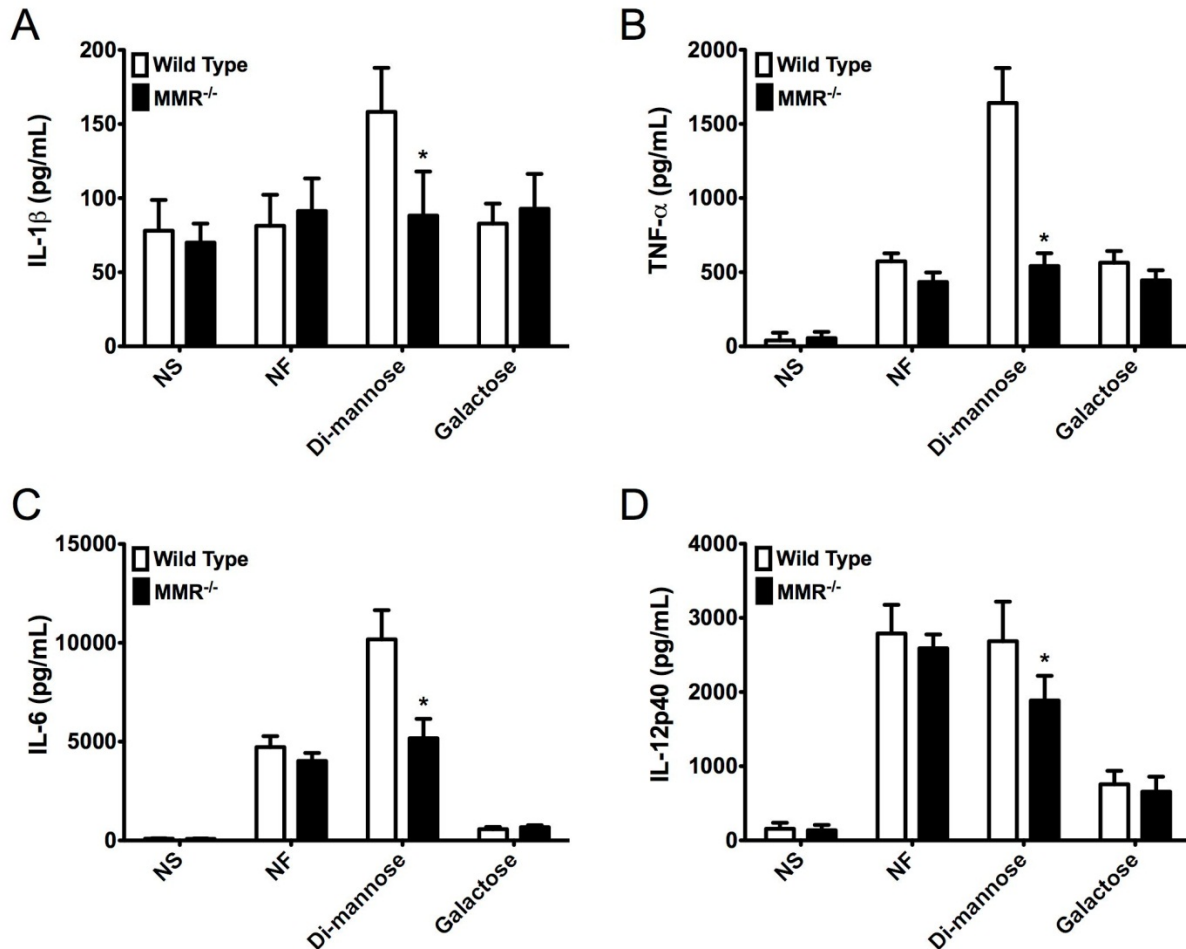
The authors would like to thank Shawn Rigby for his expertise in flow cytometry as well as the United States Army Medical Research and Materiel Command for financial support (Grant No. W81XWH-10-1-0806).



**Supplemental Figure 5.1 Di-mannose functionalized nanoparticles enhanced internalization by engaging the macrophage mannose receptor on bone marrow-derived macrophages (BMM $\square$ ).** Percent of wild type ( $\square$ ) and MMR-deficient (MMR<sup>-/-</sup>;  $\blacksquare$ ) BMM $\square$  that internalized nanoparticles after 48 hrs. Data are expressed as the mean  $\pm$  the SEM of three independent experiments performed in triplicate. \* represents a statistically significant difference between wild type and MMR<sup>-/-</sup> BMM $\square$  within a treatment at  $p < 0.05$ . # represents a statistically significant difference from the non-functionalized nanoparticle treatment group for wild type BMM $\square$ .



**Supplemental Figure 5.2 Di-mannose functionalized nanoparticles enhanced bone marrow-derived macrophage (BMMφ) expression of MHC, T cell co-stimulatory molecules and CLRs by engaging the macrophage mannose receptor.** After stimulation with non-functionalized (NF) or functionalized nanoparticles for 48 hrs, wild type (□) and MMR-deficient (MMR<sup>-/-</sup>; ■) BMMφ were harvested and analyzed by flow cytometry for surface expression of (A) MHC I, (B) MHC II, (C) CD86, (D) CD40, (E) MGL, or (F) CIRE. Data are expressed as the mean ± the SEM of three independent experiments performed in triplicate. \* represents a statistically significant difference between wild type and MMR<sup>-/-</sup> BMMφ within a treatment at p < 0.05. MFI = mean fluorescence intensity.



**Supplemental Figure 5.3 Di-mannose functionalized nanoparticles enhanced bone marrow-derived macrophage (BMDM) pro-inflammatory cytokine production by engaging the macrophage mannose receptor.** After stimulation with non-functionalized (NF) or functionalized nanoparticles for 48 hrs, culture supernatants from wild type (□) and MMR-deficient (MMR<sup>-/-</sup>; ■) BMDMs were harvested and assayed for (A) IL-1 $\beta$ , (B) TNF- $\alpha$ , (C) IL-6, and (D) IL-12p40. Non-stimulated cells (NS) were used as a negative control. Data are expressed as the mean  $\pm$  the SEM of three independent experiments performed in triplicate. \* represents a statistically significant difference between wild type and MMR<sup>-/-</sup> BMDMs within a treatment at p < 0.05.



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## CHAPTER 6: CONCLUSIONS AND FUTURE WORK

### 6.1 Conclusions

This work was focused on the design of surface functionalized polyanhydride nanoparticles as novel adjuvants by targeting C-type lectin receptors (CLRs) as a strategy to tailor the immune response for the rational design of targeted vaccines against particular pathogens. This study evaluated amphiphilic polyanhydride nanoparticles based on 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis(*p*-carboxyphenoxy)hexane (CPH) as adjuvants and targeted delivery systems. The two saccharides used to modify the surface of these nanoparticles were galactose and di-mannose, which target the macrophage galactose lectin (MGL) and the macrophage mannose receptor (MMR) respectively.

The high throughput synthesis and characterization of these functionalized nanoparticles was addressed in Chapter 4. A high throughput set up operated by LabView<sup>®</sup> was used for the synthesis of the nanoparticles and for their surface functionalization. Optimal conditions were determined in order to maintain nanoparticle morphology and a consistent and reproducible concentration of carbohydrate attached to the surface.

The evaluation of the capabilities of these functionalized polyanhydride nanoparticles to target specific CLRs on alveolar macrophages (AM $\square$ ) was presented in Chapter 5. In this work, the study of internalization and activation (i.e., cell surface marker expression, cytokine production, and reactive nitrogen species production) of AM $\square$  treated with functionalized polyanhydride nanoparticles was investigated. In

addition, mechanistic studies were performed in order to assess the engagement of CLRs in the recognition of these novel nanoparticles and the downstream events after crosslink of the CLRs with specific ligands. The results obtained provide valuable insights into the selection of appropriate functionalization motifs for the development of intranasal vaccine formulations, depending on the particular pathogen. The adjuvant capacity of polyanhydride nanoparticles and their potential to include ligands to target specific receptors provides a versatile platform for the rational design of vaccines. In addition to the multiple parameters that can be varied (i.e. polymer chemistry, particle size, antigen loading and delivery route) to optimize vaccine delivery and protective immunity, the functionalization of these particles with different ligands adds an advantage that helps tailor the immune response in an appropriate manner for the design of vaccine formulations against specific diseases.

## **6.2 Future Work**

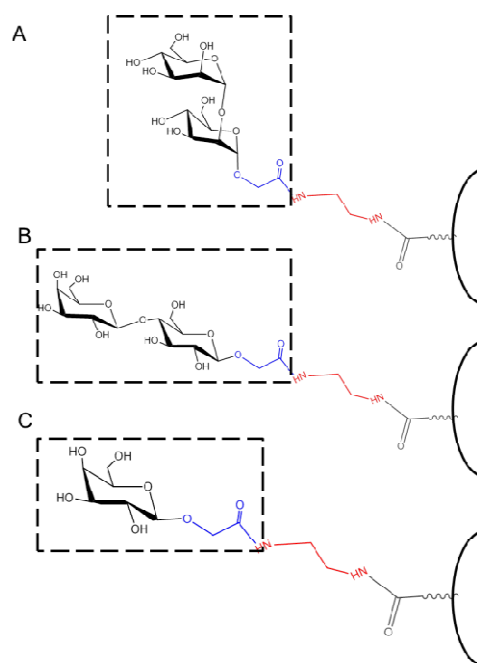
The results of this research project set the stage for the study of new applications and further investigations that can be performed with these functionalized polyanhydride nanoparticles.

### **6.2.1 Library synthesis and vaccine formulation**

As suggested before, the ligand density may be an important factor to take into consideration when targeting C-type lectin receptors. The ligand density may change the immunological potency of the resultant response [1]. This suggests that varying the amount of carbohydrate attached to the nanoparticle should be investigated to determine the effect of the density of specific ligands (i.e., di-mannose or galactose).

The high throughput set up described in Chapter 4 provides the ability to synthesize a library of functionalized polyanhydride nanoparticles by varying the amount of saccharides attached. Specific technical aspects should be investigated such as the systematic variation of the saccharide concentration on the surface of nanoparticles.

One approach is the variation of the ethylenediamine or linker (**Figure 6.1**) between the deprotected saccharide and the nanoparticles. In the protocol described in Chapter 4, 10 equivalents of ethylenediamine were employed, because an excess of equivalent was needed based on the density of carboxylic acids calculated to be present on the surface of 50:50 CPTEG:CPH polyanhydride nanoparticles [2]. By varying the number of equivalents of ethylenediamine (attached in the first reaction described in Chapter 4), the molecules available for the second reaction will change as well. An alternative approach is to change the number of equivalents of deprotected saccharides. This option may lead to a higher number of free ethylenediamine groups on the surface of the nanoparticles. In addition, a combination approach of varying both the number of ethylenediamine equivalents and saccharides can be evaluated.



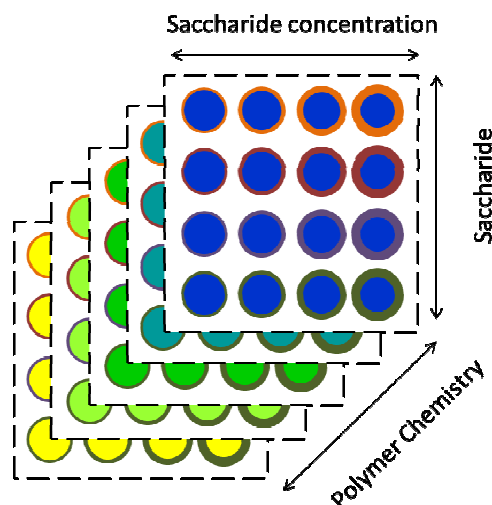
**Figure 6.1 Schematic representation of saccharides attached.** The structure of (A) di-mannose, (B) lactose, and (C) galactose attached to polyanhydride nanoparticles is represented. ( [ ] ) Highlights each desprotected saccharide, and — highlight the ethylenediamine linker used for the amine carboxylic acid coupling reaction.

Another approach is to evaluate a different method to attach the saccharide to the nanoparticle, such as a biospecific biotin-streptavidin linkage. Streptavidin, a tetrameric 52,800 Da MW protein, could be attached to the surface of polyanhydrides by an amine coupling reaction [3]. Then, different methods of producing biotinylated saccharides may be studied. This may facilitate the rapid variation of saccharides on the surface of nanoparticles.

After designing a systematic method to change the ligand density on the surface of the nanoparticles, further studies could be performed to evaluate how the ligand density affects the activation of antigen presenting cells (APCs). In addition, different chemistries of polyanhydride nanoparticles can be functionalized in order to evaluate how the chemistry and functionalization together affect the activation of APCs.



The set up described in Chapter 4 enhances the throughput of the nanoparticle synthesis and functionalization process. Therefore, a 3D library can be created by varying not only the amount of saccharides attached to the surface of the nanoparticles, but also the saccharides themselves along with the polymer chemistry (**Figure 6.2**).



**Figure 6.2 Schematic representation of the proposed 3D library.** The representation shows how a 3D library can be synthesized by simultaneously varying the saccharide, the saccharide concentration, and polymer chemistry.

Further studies can evaluate the activation of  $CD4^+$  and  $CD8^+$  T cells and their interaction with APCs treated with functionalized polyanhydride nanoparticles, in order to study how the immune response is tailored by the targeting of different CLRs. Biodistribution studies after intranasal administration of functionalized nanoparticles may provide valuable information, identifying how immune cells internalize these particles and transport them to the draining lymph nodes.

The efficacy of these functionalized polyanhydride nanoparticles may be tested *in vivo* as a vaccine formulation encapsulating an antigen against a specific disease. This

process may be challenging if the encapsulation of the antigen is performed prior to the surface functionalization by using a method such as anti-solvent nanoprecipitation. During the functionalization reactions, the nanoparticles are in aqueous environment, which causes some degradation, leading to the premature release of the antigen. Since these antigens may have amine motifs, these groups might compete with the amine carboxylic coupling reaction, potentially decreasing the amount of saccharides attached to the surface of the nanoparticles. In addition, the loading of antigen may be affected by the functionalization process.

### **6.2.2 Novel ligands targeting other CLRs**

The array of CLRs that may be targeted is wide and functionalization with different ligands is another direction that might be explored. Several ligands are recognized by a single CLR and the crosslinking with different ligands may lead to a different response of the cell [4, 5]. Several ligands should be engineered to target diverse CLRs (**Table 6.1**). It is important to emphasize that different ligands might be recognized by several CLRs, providing synergy among different CLRs causing a potent immune response. For example, mannose is recognized by MMR and CIRE receptor and N-acetylglucosamine is recognized by MMR and the Endo-180 receptor, and a combination of mannose- and N-acetylglucosamine may produce a different downstream response upon crosslinking with these receptors. The outcome after crosslinking between ligands and receptors could be varied. Some studies suggest that the Endo-180 receptor enhances the internalization of glycosylated ligands to endosomal compartments, and the downstream outcome of this observation has not

been determined [6]. The biological effect of crosslinking  $\beta$ -glucan and Dectin-1 receptors has been studied and it has been shown that both cell surface marker expression and production of cytokines such as IL-4 and IL-12 are enhanced [7-9].

**Table 6.1 Rational selection of ligands to target specific CLRs**

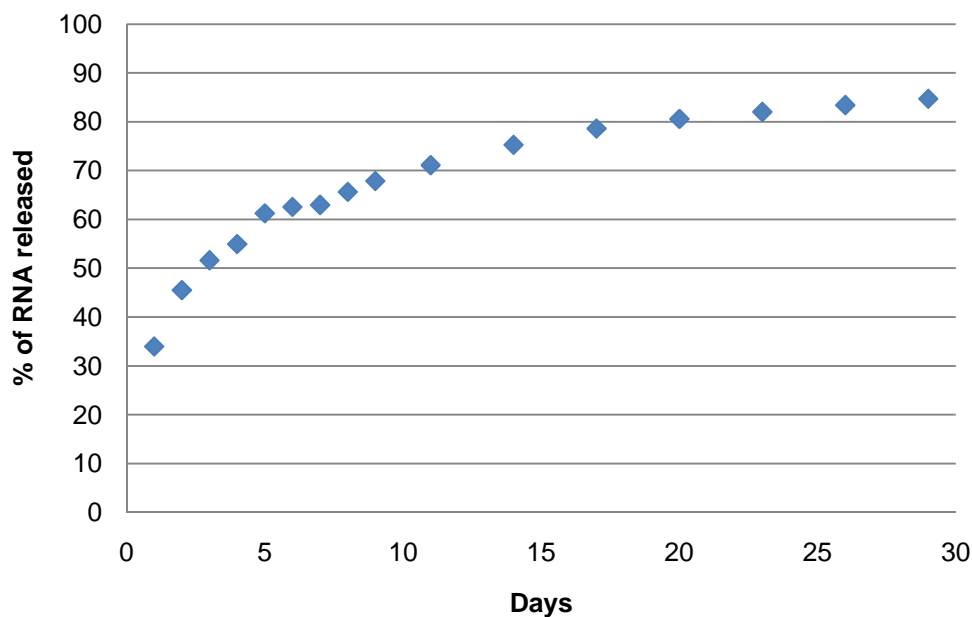
<b>Ligand</b>	<b>CLRs that might be targeted</b>
<b>Fucose</b>	MMR, Endo-180 receptor
<b>N-acetylglucosamine</b>	MMR, Endo-180 receptor, Langerin
<b>High mannose structures</b>	MMR, CIRE receptor
<b><math>\beta</math>-glucan</b>	Dectin-1
<b>N-acetylgalactosamine</b>	MGL2
<b>Mannose 6 phosphate</b>	M6P Mannose 6 phosphate receptor

### **6.2.3 Novel applications: from gene delivery to cancer vaccines**

The diverse outcomes obtained with different functionalized nanoparticles may provide a versatile platform that could be used for several applications beyond vaccine design. It is known that several strategies (liposomes, mannose-DNA complexes, etc.) targeting the mannose receptor have enhanced the delivery of genes into macrophages [10-13]. These insights may be useful to design a DNA vaccine adjuvant by combining the adjuvant properties of polyanhydride nanoparticles with targeting using MMR to improve transfection efficiency. It is well known that MMR leads to the internalization of a glycosylated antigen into the endosomal compartment. As a consequence, a limitation of transfecting genes with this approach may be DNA degradation in endosomal compartments and its escape from this compartment. New strategies that could be used to promote the escape of DNA or RNA molecules from the endosome include the

addition of endosomolytic components such as adenovirus particles and influenza peptides [10].

We have performed some preliminary work in this area by encapsulating an RNA molecule (i.e., 3UP8C3PEG molecule provided by Joonbae Seo from Dr. Ravi Singh's laboratory at ISU) and studying its release from 50:50 CPTEG:CPH nanoparticles (**Figure 6.3**). Further *in vivo* and *in vitro* studies may be performed with functionalized nanoparticles in order to assess the capacity of these nanoparticles to transfect DNA or RNA molecules.



**Figure 6.3 RNA release from 50:50 CPTEG:CPH nanoparticles.** The release study was performed at constant agitation at 37°C in an aqueous buffer for 30 days.

Another application that can be studied is the development of cancer vaccines using functionalized polyanhydride nanoparticles. It is known that the targeting of the mannose 6 phosphate (M6P) receptor (a receptor localized as a transmembrane protein in the Golgi) provides tumor suppressor properties [14]. A similar capability has been attributed to the targeting of MGL. Transformation or glycan modifications at the cell surface may provide oncogenic signaling (tumor transformation, invasion and metastasis) [15]. MUC1 glycoprotein is an example of a protein present on the surface of mammary cells, whose expression is increased on breast cancer cells. This is a tumor-associated glycoform that can be recognized by MGL by promoting its internalization and presentation on MHC I and II complexes. This specific recognition and presentation has positive implications for the design of cancer vaccines [16, 17]. The tumoricidal cellular recognition in addition to enhancement of the uptake of glycosylated antigens are important attributes that make galactose-functionalized nanoparticles promising candidates for the development of cancer vaccines.

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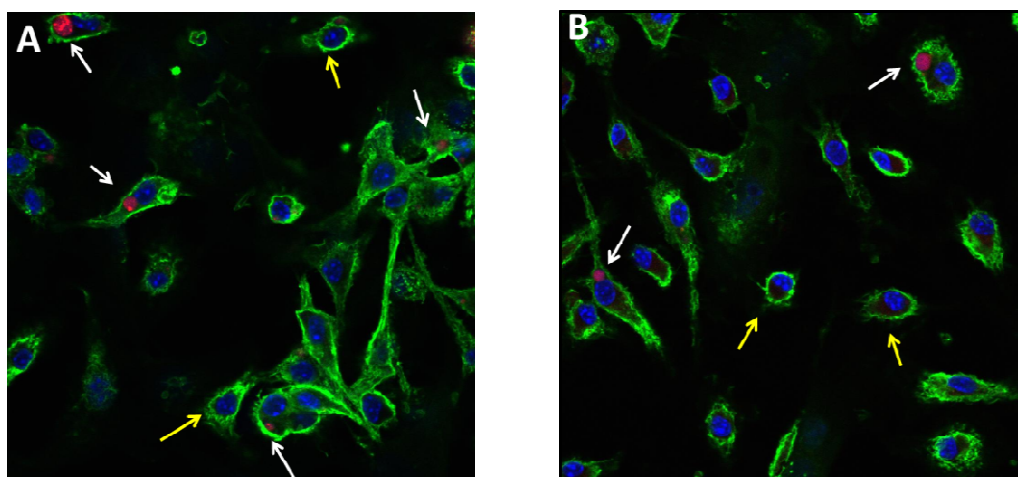
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## APPENDIX: TAILORING THE IMMUNE RESPONSE OF BONE MARROW DERIVED MACROPHAGES

Section to be submitted to Biomaterials as supplementary data

### A1.1 Internalization assessment

The internalization of quantum dot loaded functionalized nanoparticles was evaluated using confocal microscopy. Cell membrane was stained using Oregon Green® 488 conjugated WGA (Invitrogen) and images corroborate the actual internalization of nanoparticles as supposed to particles associated to cell membrane (**Figure A1.1**). These results validate the technique to quantify internalization of nanoparticles using flow cytometry.



**Figure A1.1 Confocal Microscopy qualitative assessment of internalization.** In order to determine the role of internalization of nanoparticles, QDs loaded nanoparticles were used to stimulate BMM (A) and AM (B) harvest on wild type B6 mice. Yellow arrow show cells that did not internalize nanoparticles and white arrows highlight internalized nanoparticles. Prior staining for flow cytometry analysis, some samples were separated and stained for confocal microscopy analysis verifying that QDs loaded nanoparticles were actually internalized by macrophages. Cells were fixed on glass coverslips and stained with Oregon Green® 488 conjugated WGA (Invitrogen) following the procedure recommended by the provider. Coverslips were mounted using ProLong Gold with DAPI mounting medium (Invitrogen).



## A1.2 Cell surface marker expression and cytokine production

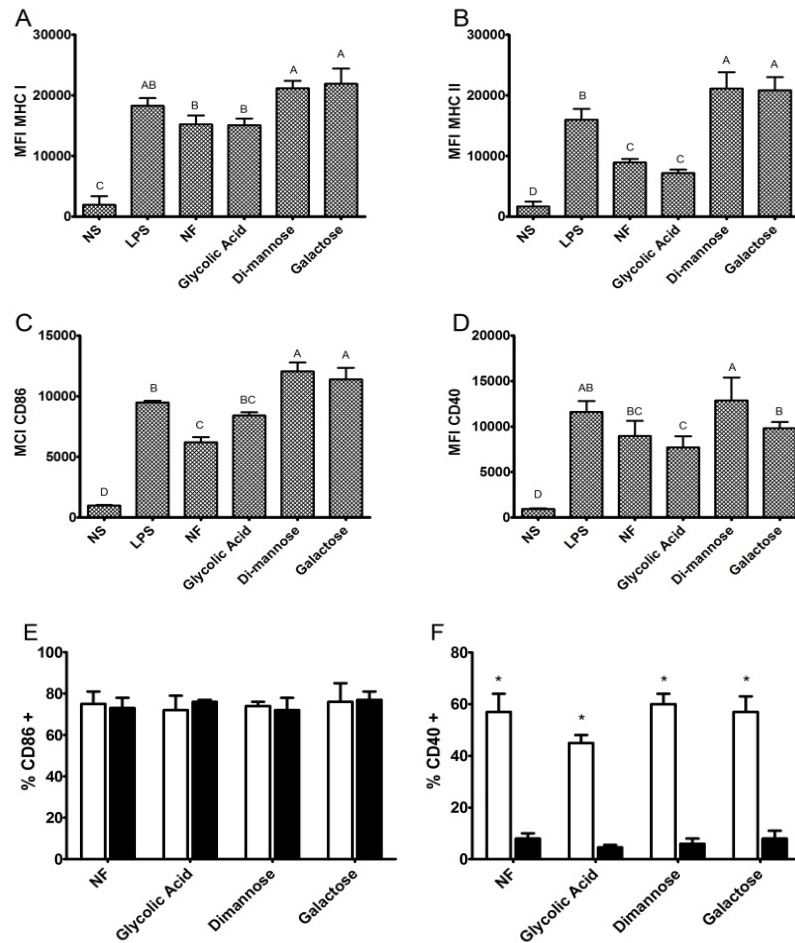
Similarly to the results presented in **Chapter 5**, the activation of bone-marrow derived macrophages (BMM $\phi$ s) was evaluated measuring cell surface marker expression and cytokine production. The results herein presented are similar to the ones obtained on **Chapter 5** where AM $\phi$  were the main subject of investigation.

The expression of MHC I and MHC II is fundamental for antigen presentation. The stimulation of BMM $\phi$ s with galactose and di-mannose functionalized nanoparticles resulted in an up-regulation in the expression of these two markers. The expression of co-stimulatory molecules, i.e. CD40 and CD86, needed for the activation of T cells, was enhanced as well by galactose and di-mannose functionalized polyanhydride nanoparticles in comparison to the non-functionalized group (**Figure A1.2**). Internalization resulted required for the expression of CD40, will no difference was observed in the percentage of cells positive for MHC I, MHC ii and CD86 and positive for nanoparticles, suggesting that only interaction of cells with particles, as well as the microenvironment generated by stimulation, was sufficient for the expression of these markers (**Figure A1.2**). The expression of CLRs was enhanced depending on the stimulation group. The expression of the macrophage mannose receptor (MMR) was enhanced by di-mannose functionalized nanoparticles; similarly this group was the main group that enhanced the expression of CIRE receptor. The macrophage galactose lectin (MGL) was enhanced mainly by galactose-functionalized nanoparticles (**Figure A1.3**). These results suggest that the target of these specific CLRs improved the expression of markers needed for antigen presentation and T-cell activation.

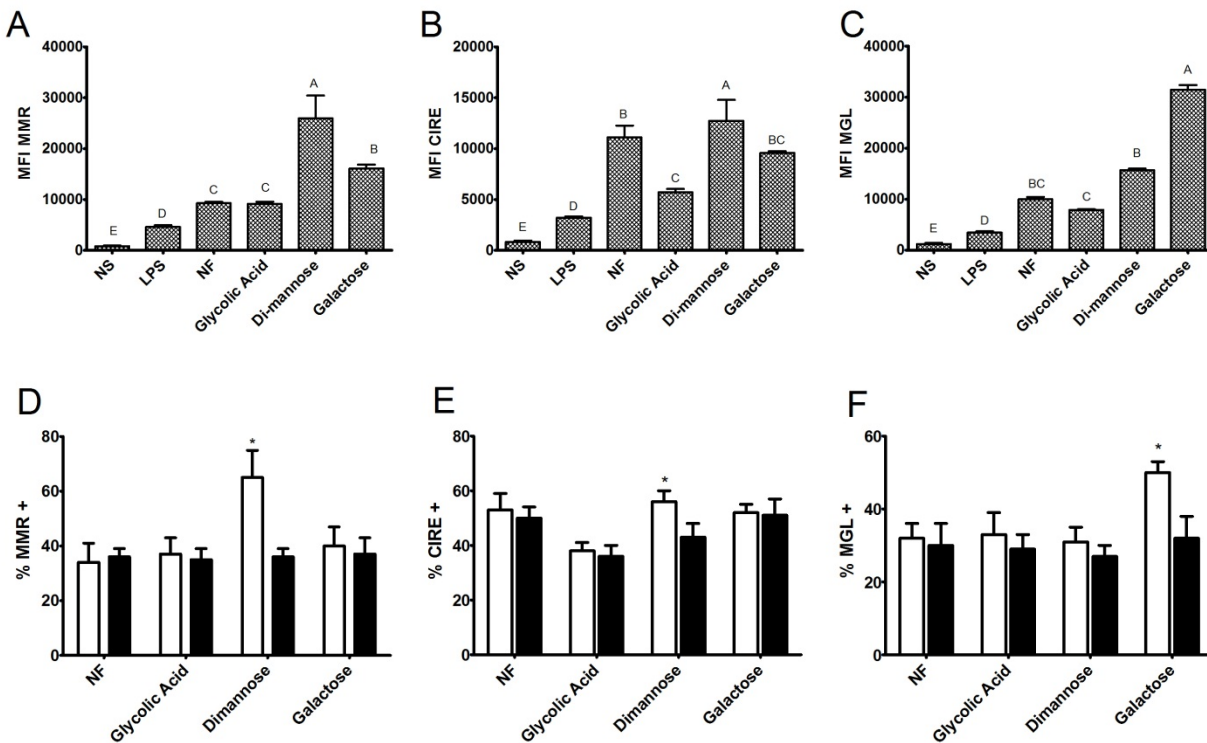
The internalization of specific functionalized nanoparticles enhanced the expression of specific CLRs, since there was a significant difference in the percentage of cells double positive for a specific CLR and nanoparticles depending on the functionalization (**Figure A1.3**). The Internalization of di-mannose functionalized nanoparticles enhanced the expression of MMR and CIRE receptor, while the internalization of galactose functionalized nanoparticles up regulated the expression of MGL. These results suggest that the internalization of nanoparticles was effectively mediated by specific receptors depending on the functional groups located on the surface of nanoparticles.

The presence or absence of cytokines is fundamental to tailor the immune response. The production of pro-inflammatory cytokines, IL-12p40 and IL-10 was measured on the supernants of stimulated cells. The production of pro-inflammatory cytokines (i.e. IL-6, IL-1 $\beta$  and TNF- $\alpha$ ) was enhanced mainly by di-mannose functionalized nanoparticles compared with the non-functionalized group, but no significant amounts of reactive nitrogen species (RNS) were produced by BMM $\phi$  under any stimulation treatment (**Figure A1.4**). This results suggest that the simulation with di-mannose functionalized nanoparticle are able to induce a secondary signal (pro-inflammatory cytokines) for the activation of T cells. The production of IL-12p40 (which main function is direct the immune response towards a Th1 pathway) was enhanced as well by the di-mannose functionalization, and the galactose functionalization resulted in a down regulation on its production (**Figure A1.4**). There was no secretion of IL-10 under any stimulation group (data not shown). These results suggest that even when

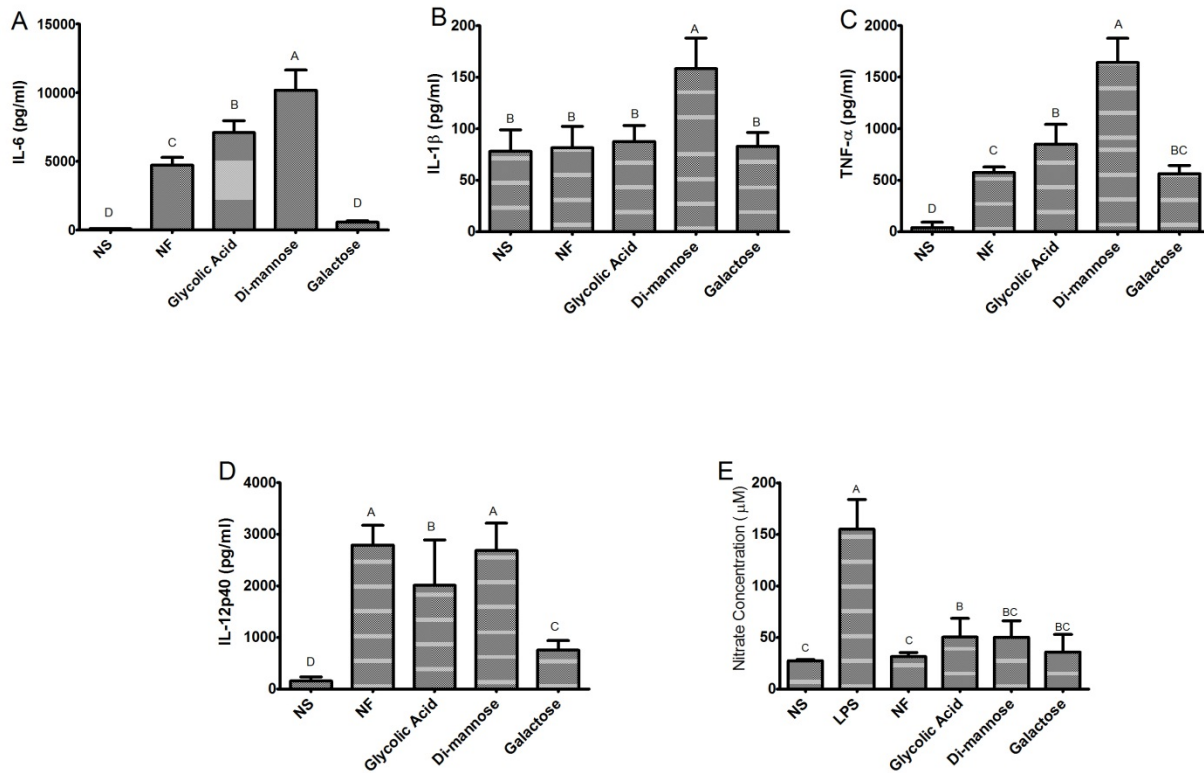
CIRE, MMR and MGL are CLRs that mediate the internalization of antigens, different immune outcome can be generated depending on the CLR that is targeted.



**Figure A1.2 Cell surface expression of activation markers.** Bone-marrow derived macrophages (BMMφs) harvested from wild type B6 mice were stimulated with non-functionalized (NF) nanoparticles and different functionalized nanoparticle treatments. Non-stimulated (NS) and LPS-stimulated groups were included as negative and positive controls, respectively. After 48 h, cells were stained and analyzed by flow cytometry for surface expression of (A) MHC I, (B) MHC II, (C) CD86, and (D) CD40. Di-mannose and galactose modified nanoparticles effectively enhanced the expression of MHC I, MHC II, CD86 and CD40 compared with the NF nanoparticles. In order to determine the role of nanoparticle internalization, QD-loaded nanoparticles were used to stimulate BMMφ. Percentage of cells positive for (E) CD86 and (F) CD40 were identified in nanoparticle<sup>+</sup> (double positive, □) and nanoparticle<sup>-</sup> (positive for marker/negative for nanoparticle, ■) populations. The mean ± standard error of three independent experiments performed in triplicate is shown. MFI = mean fluorescent intensity. Different letters indicate statistical differences among treatment groups (Tukey HSD test,  $p < 0.05$ ). (\*) represents statistically significant differences between nanoparticle<sup>+</sup> and nanoparticle<sup>-</sup> groups (T-test,  $p < 0.05$ ).



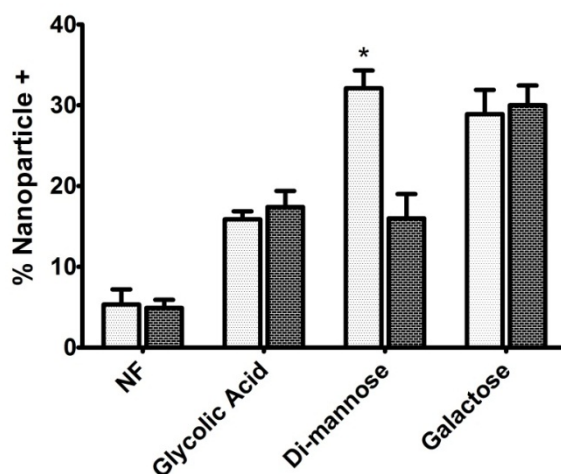
**Figure A1.3 Cell surface expression of CLR receptors.** Bone-marrow derived macrophages (BMM $\phi$ s) harvested from wild type B6 mice were stimulated with non-functionalized (NF) nanoparticles and different functionalized nanoparticle treatments. Non-stimulated (NS) and LPS-stimulated groups were included as negative and positive controls, respectively. After 48 h, cells were stained, and analyzed by flow cytometry for surface expression of (A) MMR, (B) CIRE receptor, and (C) MGL. The data indicate that while di-mannose functionalization enhanced the expression of MMR and galactose functionalized nanoparticles enhanced the expression of MGL, the expression of CIRE receptor by the different functionalization groups was similar to that of the NF group. In order to determine the role of nanoparticle internalization, QD-loaded nanoparticles were used to stimulate BMM $\phi$ . Percentage of cells positive for (D) MMR, (E) CIRE, and (F) MGL were identified in nanoparticle<sup>+</sup> (double positive,  $\square$ ) and nanoparticle<sup>-</sup> (positive for marker/negative for nanoparticle,  $\blacksquare$ ) populations. The mean  $\pm$  standard error of three independent experiments performed in triplicate is shown. MFI = mean fluorescent intensity. Different letters indicate statistical differences among treatment groups (Tukey HSD test,  $p < 0.05$ ). (\*) represents statistically significant differences between nanoparticle<sup>+</sup> and nanoparticle<sup>-</sup> groups (T-test,  $p < 0.05$ ).



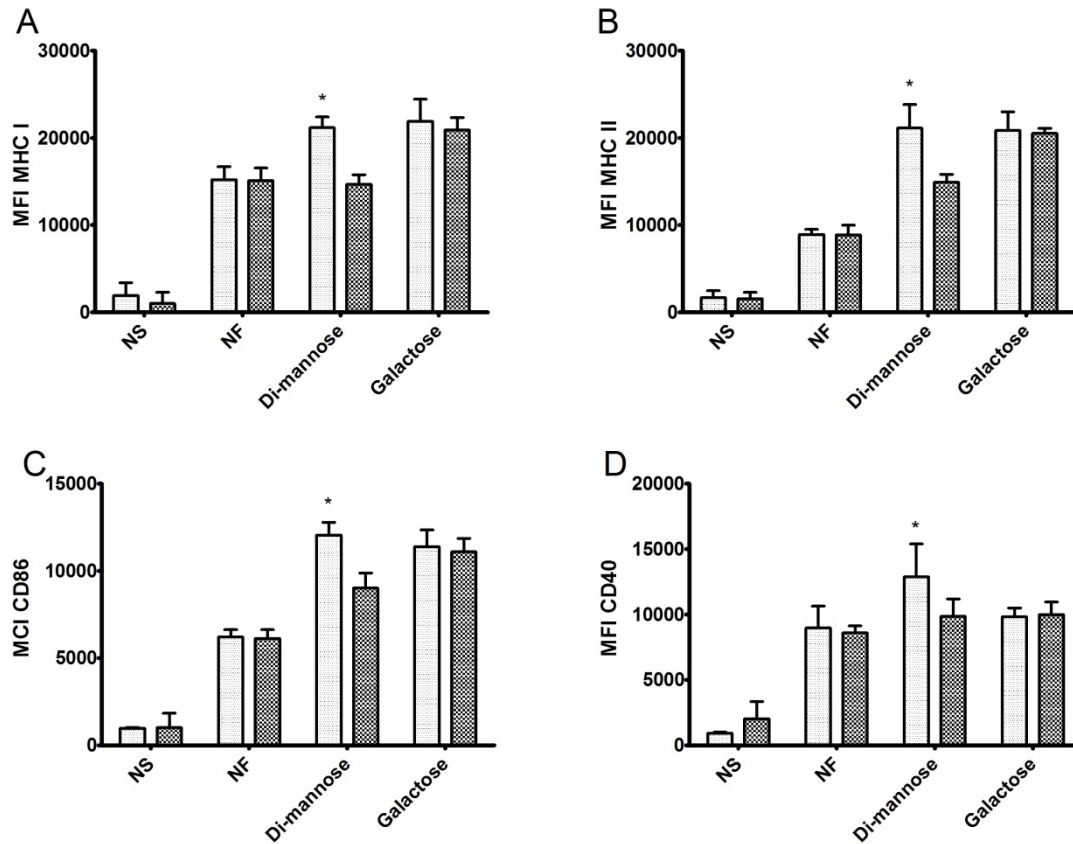
**Figure A1.4 Cytokine production and reactive nitrogen species quantification.** Bone-marrow derived macrophages (BMMs) harvested from wild type B6 mice were stimulated with non-functionalized (NF) nanoparticles and different functionalized nanoparticle treatments. Non-stimulated (NS) and LPS-stimulated groups were included as negative and positive controls, respectively. After 48 h, supernatants were collected and used to measure the concentration of the pro-inflammatory cytokines (A) IL-6 (B) IL-1 $\beta$ , (C) TNF- $\alpha$ , as well as (D) IL-12p40 and IL-10. In these studies, IL-10 was not detected for any of the treatments (data not shown). BMMs stimulated with LPS induced the following amounts of cytokines: 10,194.80  $\pm$  148.9 pg/mL for IL-1 $\beta$ , 95,787.70  $\pm$  175.05 pg/mL for IL-6, 7,853.5  $\pm$  122.18 pg/ml for TNF- $\alpha$ , and 4,465.33  $\pm$  134.69 for IL-12p40). (F) Nitrate concentration was measured on supernatants by a Griess assay as an indirect method of quantification of reactive nitrogen species. The mean  $\pm$  standard error of three independent experiments performed in triplicate is shown. Different letters entail statistical differences among treatment groups (Tukey HSD test, p < 0.05).

### A1.3 Engagement of MMR

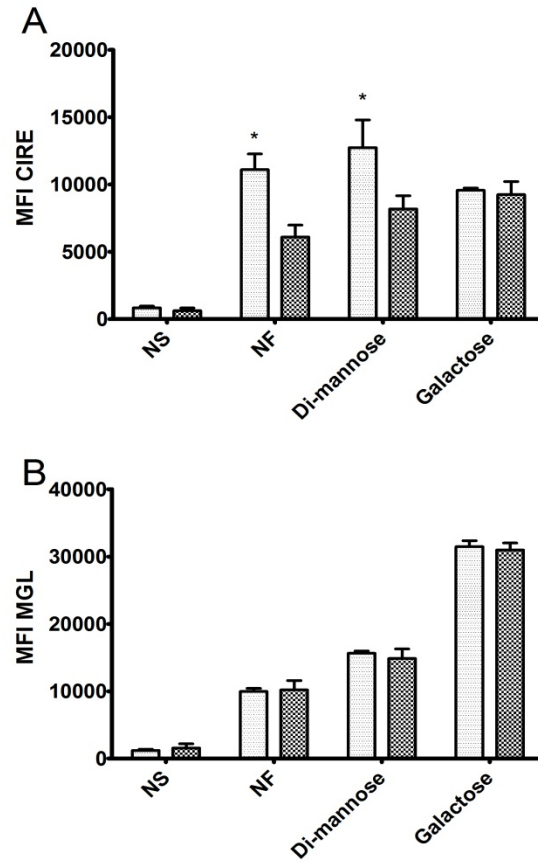
The engagement of the MMR in the recognition of di-mannose functionalized nanoparticles was corroborated using BMM $\phi$  derived from bone-marrow of macrophage mannose receptor knock out (MMR KO) mice. Statistical differences was observed not only in the rate of internalization of particles (**Figure A1.5**), but also in the expression of cell surface markers (i.e. activation markers and CLRs) (**Figures A1.6 and A1.7**), and the production of cytokines (**Figure A1.8**) only when MMR KO BMM $\phi$  were stimulated with the di-mannose functionalized polyanhydride nanoparticles.



**Figure A1.5 Internalization of functionalized and non-functionalized nanoparticles.** Bone-marrow derived macrophages (BMM $\phi$ s) were stimulated with QD-loaded functionalized and non-functionalized (NF) nanoparticles. After 48h, cells harvested from wild type (■) or MMR KO mice (■) were stained and analyzed by flow cytometry. Percentage of BMM $\phi$  that internalized nanoparticles was measured as percentage of cells that were positive for QD-loaded functionalized and non-functionalized nanoparticles (with appropriate background subtraction). The mean  $\pm$  standard error of three independent experiments performed in triplicate is shown. (\*) represents statistically significant differences between BMM $\phi$  harvested from wild type or MMR KO mice (T-test,  $p < 0.05$ ).

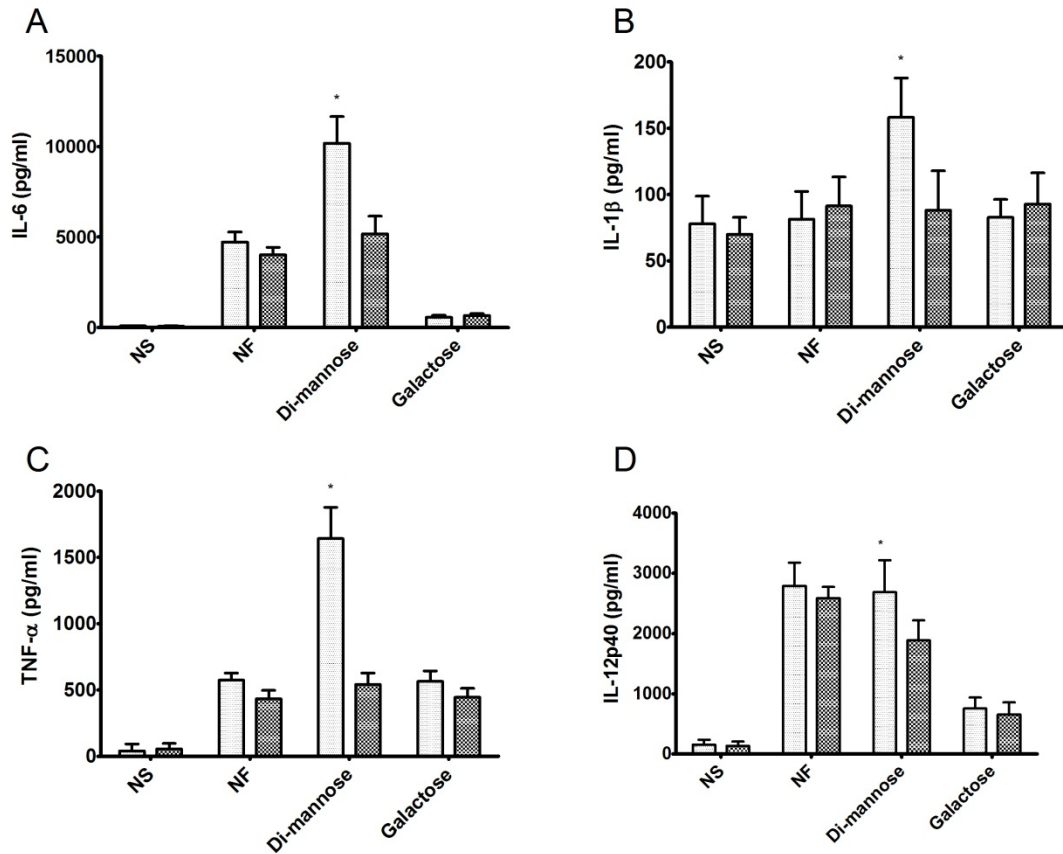


**Figure A1.6 Effect of the knockout of MMR on cell surface marker expression of activation markers.** Bone-marrow derived macrophages (BMM $\phi$ s) were stimulated with QD-loaded functionalized and non-functionalized (NF) nanoparticles. After 48h, cells harvested from wild type (■) or MMR KO mice (▨) were stained and analyzed by flow cytometry for surface expression of (A) MHC I (B) MHC II, (C) CD86 and (D) CD40. The mean  $\pm$  standard error of three independent experiments performed in triplicate is shown. MFI = mean fluorescent intensity. (\*) represents statistically significant differences between BMM $\phi$  harvested from wild type or MMR KO mice (T-test,  $p < 0.05$ ).



**Figure A1.7 Effect of the knockout of MMR on the expression of CLRs.** Bone-marrow derived macrophages (BMMs) were stimulated with QD-loaded functionalized and non-functionalized (NF) nanoparticles. After 48h, cells harvested from wild type (■) or MMR KO mice (▨) were stained and analyzed by flow cytometry for surface expression of (A) CIRE receptor and (B) MGL. The mean  $\pm$  standard error of three independent experiments performed in triplicate is shown. MFI = mean fluorescent intensity. (\*) represents statistically significant differences between BMMs harvested from wild type or MMR KO mice (T-test,  $p < 0.05$ ).





**Figure A1.8 Effect of the knockout of MMR on the production of cytokines.** Bone-marrow derived macrophages (BMMs) were stimulated with QD-loaded functionalized and non-functionalized (NF) nanoparticles. After 48h, supernants were collected to measure cytokine concentration on AM cultures harvested from wild type (■) or MMR KO mice (▨). The mean  $\pm$  standard error of three independent experiments performed in triplicate is shown. (\*) represents statistically significant differences between BMM harvested from wild type or MMR KO mice (T-test,  $p < 0.05$ ).

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- Chávez-Santoscoy, A.V.; Huntimer, L.; Wannemuehler, M.; Narasimhan, B.; "Harvesting Alveolar Macrophages to Evaluate Polyanhydride Nanoparticles Activation *In vitro*". **Accepted** JoVE: Journal of Visualized Experiments.
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- Carrillo- Conde, B.; Song E.; Chávez-Santoscoy, A.V.; Pohl, N.; Wannemuehler, M.; Narasimhan, B.; "Mannose Functionalized "Pathogen-Like" Polyanhydride Nanoparticles Target C-Type Lectin Receptors on Dendritic Cells", **Accepted** *Molecular Pharmaceutics*, 2011.
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- Baylon-Cardiel, J., Jesús-Pérez, N.M.; Chávez-Santoscoy, A.V., Lapizco-Encinas, B.H.\*; Controlled microparticle manipulation employing low frequency alternating electric fields in an array of insulators, in press, *Lab on a Chip*, 2010. I.F. 6.342. DOI:10.1039/C0LC00097C.

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